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14. ABSTRACT

One of the hallmarks of cancer is the deregulation of the cell proliferation. This deregulation promotes genetic errors that contribute to genomic instability. Our hypothesis is that Cdk2 exists in two freely exchangeable conformations: that seen in the active, cyclin—bound crystal and that of the inactive monomeric Cdk2, with the latter predominating in the absence of cyclin. We propose that phosphorylation of Cdk2T39 shifts the equilibrium in the direction of the active conformation that best fits cyclin and therefore facilitating cyclin binding, G1 progression and initiation of DNA synthesis. We will test this hypothesis by treating recombinant cdk2 with AKT and sending it for mass spectroscopy so we can determine if Cdk2 is indeed phosphorylated by AKT. We will also determine the effect of AKT phosphorylation on Cdk2 by constructing a phosphomimetic mutant of Cdk2 and determining if this has an effect on cyclin binding and G1 progression. Ultimately, this research may elucidate a novel method of cell cycle control through which mitogenic signals may influence the cell cycle.

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TABLE OF CONTENTS

INTRODUCTION

For the past three years, we have been dissecting the role of CDK2T39 phosphorylationof CDK2 function and cell cycle progression. Our previous work had established that CDK2 is phosphorylated at threonine 39 by AKT *in-vitro* and that Immunoprecipitated CDK2 reacts with an anti-phospho AKT substrate antibody and that this reactivity is lost after inhibition of the PI3K signaling pathway – thus suggesting that CDK2 is phosphorylated by AKT *in vivo*. Additionally we had preliminarily data suggesting that phosphorylation of CDK2 at threonine 39 influences cyclin binding. Using budding yeast as a model system we have also shown that alteration of this site has an effect on cell cycle progression. Namely, replacing the endogenous CDK2 homologue, Cdc28, with Cdc28S46E leads to a decrease in the G1 phase transit time. Here we report that we have used an *in vitro* protein binding assay to show the rate of cyclin-CDK2 binding is influenced by phosphorylation on CDK2T39 and that in yeast cdc28S46E has greater kinase activity than Cdc28. Altogether our data shows that phosphorylation of this AKT substrate site in cyclin dependent kinases affects the rate of cyclin-CDK complex formation, catalytic activity and S phase entry time.

BODY

Mutations affecting the Cdk2T39 site alter cyclin binding *in vitro*

Our preliminary data suggested that phosphorylation of CDK2T39 may affect cyclin binding (for a thorough review, please see the appendix 2). Those experiments, however, did not allow the binding to reach a saturation state and thus it was impossible

to determine if the rate of association was the only factor affected or if there was an increase in steady state binding (lower Kd). Additionally our preliminary data did not contain appropriate controls to mitigate the possibility that AKT may be acting as a scaffold protein and thus aiding the cyclin-CDK complex formation. Therefore, we decided to test effects of Cdk2T39 phosphorylation on cyclin binding more thoroughly. Recombinant cyclin A or cyclin E proteins were incubated with their respective antibodies (see appendix 2) and complexes pre-bound to Protein A sepharose beads and washed extensively to remove unbound cyclin. Equal inputs of the different recombinant Cdk2 (WT, T39A and T39E) were then added to the pre-loaded protein A sepharose beads-antibody-cyclin complex for the times shown and then cyclin-bound Cdk2 was assayed by immunoblotting after the complexes were resolved by SDS-PAGE and transferred (IP-blots shown for cyclin A-Cdk2 in Figure 1A). For both cyclin A and Cyclin E- bound Cdk complexes, Cdk2T39E reached a maximal level of binding sooner than Cdk2wt (Figure 1 B). Cdk2T39E reached maximal cyclin A binding in less than 150 seconds whereas steady state Cdk2wt binding was reached by 10 minutes (graphed as % input binding in Figure 10B-representative of three experiments). Similarly, the time required for maximal Cdk2T39E binding to cyclin E was also shorter than Cdk2wt (Figure 1C). All Cdk2 isoforms reached a similar maximal level of binding, indicating that this phosphorylation may not be required for binding and that this phosphorylation does not change the steady state binding of cyclin-CDK complexes (does not lower Kd). What is clear from our in vitro data is that this phosphorylation speeds up the rate of the reaction.

Cdk2 phosphorylation by Akt increases the cyclin-Cdk2 assembly rate in vitro.

To further assay how phosphorylation of Cdk2pT39 affects the rate of cyclin-Cdk2 association, recombinant Cdk2 was reacted with active Akt for 60 minutes prior to incubation with recombinant cyclin A for intervals between 1 and 30 minutes. Cyclin A-bound Cdk2 was assayed by cyclin A immunoprecipitation followed by immunoblotting for

Cdk2. Akt treatment increased the rate of cyclin A-Cdk2 complex formation. Two different controls were used: recombinant Cdk2 was mock treated without addition of Akt (untreated), and in addition, a second Cdk2 samples was treated with catalytically inactive Akt (produced by boiling active Akt for 30 min). Cdk2 binding to cyclin occurred at similar rates in both control experiments, while pre-treatment of Cdk2 with active Akt shortened the time required for maximal cyclin A-Cdk2 association (Figure 2). It is noteworthy that all binding assays reached a maximum by 30 minutes, with comparable steady state complex detected at these concentrations of input cyclin and Cdk2. Data from Figures 1 and 2 together support the conclusion that Cdk2 phosphorylation by Akt augments the rate of cyclin binding or impairs its dissociation, once bound.

Testing effects of Cdc28-S46 mutations on Cyclin-CDK catalytic activity.

The AKT substrate site is highly conserved in cyclin dependent kinases. Earlier work had demonstrated that this homologous site in the budding yeast is indeed phosphorylated in vivo[25,26]. Cdc28, the only G1 Cdk in the budding yeast, is an essential gene that encompasses the functions of Cdk1 and Cdk2 in higher eukaryotes. Beyond the simplified cell cycle components, yeast is a good model system in which to study the cell cycle because of the relative ease with which the cells can be synchronized in G1 and the short doubling time.

In my previous report, I described the development of yeast strains that contained different isoforms of Cdc28 and how cdc28S46E showed a faster G1 ransit time. As proposed in my last report, I used these strains to assay timing of Cln2-Cdc28 kinase activativvity at specific intervals after alfa factor release from quiescence until S phase. We determined the Cdc28 kinase activity of strains reported in Table 1. Lysates from early log phase cells were used to immunoprecipitate Cdc28 and compare the their activity using histone H1 as substrate. As shown in Fig 3, equal amounts of Cdc28 were immunoprecipitated from each strain (Fig 3 top). The kinase activity of Cdc28-S46E

mutant was significantly higher compared the strains that Cdc28 wt and cdc28-S46A. This difference in catalytic ability may be the reason that cdc28-S46E triggers an early onset into S phase. To test this hypothesis, we tested if cdc28-S46E mutants had a higher catalytic ability throughout the cell cycle. Here again, cdc28-S46E showed a higher kinase activity in every timepoint collected (Figure 4). Although we only immunoblotted for CLN2, one cannot discount that some of the catalytic ability evidenced by the phosphorylation of histone H1 could come from Cdc28 bound to other CLNs – such as CLN1 and CLN3.

Altogether we have shown that Cdk activity is regulated by a novel site which can be acted upon by Akt. This, in turn, is a novel mechanism through which extra cellular mitogenic signals can influence cell cycle progression. Phosphorylation of this site is an early event in cell cycle progression. This phosphorylation alters the rate of cyclin binding and shortens the G_1 transit time.

DISCUSSION FROM RESULTS OBTAINED IN THE PAST THREE YEARS

The present study has revealed a novel mechanism through which the PI3K pathway may act to promote G_1 cell cycle progression through phosphorylation of T39 in Cdk2. This PI3K-dependent phosphorylation of Cdk2 at an Akt substrate motif surrounding T39 may occur in both normal and malignant cells. PI3K activity is ubiquitous to both cell types and has been shown to be required for G_1 to S progression in cell of lymphoid, fibroblast and epithelial origins[13]. Deregulation of this particular mechanism may be particularly germane to cancer, since the frequent activation of the PI3K pathway seen in many cancers [34] would drive Cdk2 T39 phosphoryation to accelerate G_1 progression.

According to the classical model of cell cycle progression, the timed sequential activation of different cyclin-Cdk complexes drives a cell to replicate its DNA and ultimately divide into two. The phosphorylation of specific substrates is critical in maintaining the ordered

sequence of events required for DNA replication and subsequent chromosomal segregation. Recent genetic experiments however have challenged the notion that specific cyclin-Cdks can only drive defined phases of the cell cycle.

Although activation of Cdk4 and Cdk6 normally precedes that of Cdk2 in G_1, knockout studies in mice have shown that these kinases are not essential for G_1 cell cycle progression in most cell types. Mouse embryos develop normally until mid gestation without all interphase Cdks [28]. Pertinent to the present work, Cdk2, whose disruption causes G_1 arrest in somatic cells[32 33] was found to be dispensible for G_1 progression in murine embryogenesis [1,20]. It is noteworthy that of all the Cdks, Cdk1 appears essential. Knockout of Cdk1 is not compensated by the presence of interphase Cdks. *CDK1*P$^{-/-}$P cells are not viable and embryos do not develop beyond the two-cell stage [28]. Moreover, replacement of *Cdk1* by *Cdk2* using homologous recombination also results in early embryonic lethality, indicating that Cdk1 cannot be compensated for by Cdk2, even when expressed from the Cdk1 locus [29].

A similar pattern was observed in cyclin knockout experiments. Whereas ablation of individual, or all members of different G_1 cyclin families lead to tissue specific developmental defects [4 7 23], knockout of S-G2-M cyclins, cyclins A2 and B1 lead to early embryonic lethality [18] consistent with their requirement for proper Cdk1 activation [2]. Altogether, these observations imply that Cdk1 is sufficient to drive cell division in most cellular lineages. However it would be wrong to suggest that interphase Cdks are dispensable for the faithful division of all cell types.

Regardless of the function that interphase Cdks may have during embryogenesis in genetically modified mice, data from somatic cells, many of which were lines derived from cancerous tissues, suggest something different. While in cultured cells derived from glioblastomas and osteosarcomas, inhibition of Cdk2 prevents proliferation [33 31], *CDK2P*$^{-/-}$Pmice do not show alterations in their brains or connective tissues [20]. Whereas the developing embryo appear capable of using Cdk1 to compensate for the genetic lack of *CDK2*, disruption of Cdk2 action in somatic cells has major consequences and results in

cell cycle arrest or death. Data from cultured cells in which Cdk2 and its partner cyclins, cyclins E and A were inhibited, indicate that these cells are dependent on normal function of G_1 Cdks for cell cycle progression. Antibody microinjection experiments indicate that interference with either cyclin E1 action in normal fibroblasts [19], or cyclin A in Hela cells [21] leads to a loss of cell cycle progression. Similarly, depletion of Cdk2 in cultured fibroblasts [22] and the expression of a dominant negative form of Cdk2 both led to G_1 arrest [33].

Malignant tumor progression appears to select for robust Cdk activities during cancer development. Deregulation of Cdk4 and Cdk6 activities have been implicated in a wide variety of tumors (including sarcoma, breast, lymphoma and melanoma) [14 20]. *CDK2* gene amplification and Cdk2 overexpression have been documented in primary colorectal [12], lung [35] and ovarian carcinomas [15]. Misregulation of D-type cyclins and overexpression of E-type cyclins are common features in many types of tumors [10]. More recent data suggests that aberrant activation of specific interphase Cdks may indeed be required for neoplasia. Cdk4-null mice, unlike their wild type counterparts, do not develop skin tumors induced by Myc [17] and are resistant to mammary tumors expressing Erbb2 and Hras under the control of the mouse mammary tumor virus promoter [36 24]. The data on cell cycle deregulation and cancer suggests that G_1 Cdks could indeed be a target for therapeutic treatment. This possibility, however, requires a better understanding of the regulatory mechanisms that underlie Cdk function.

The Akt substrate motif is conserved in other Cdks (including Cdk1 and Cdk4), indicating that aberrations in PI3K signaling could potentially augment not only Cdk2 activation but also promote the activation of other Cdks and thereby have global effects on both G_1 and G2-M progression. The specific roles of phosphorylation of homologous sites on activation of other Cdk complexes has not been assayed in our study but may follow mechanisms similar to those described herein for Cdk2.

The present study does not establish that Cdk2T39 is an exclusive target of Akt. We observed that Akt and Cdk2 form a complex in cells and that Cdk2 can be phosphorylated

at T39 by active Akt *in vitro*. The phosphorylation of Cdk1 in the homologous site (Cdk1-S39) had been previously described in human cells although the authors did not define the timing of this event nor how it may affect the cell cycle progression[27]. The S39 site in Cdk1 was shown to be phosphorylated in vitro by casein kinase II (CKII) [27]. Although CKII activity is periodically activated in early G_1 and this kinase could play a role in T39 phosphorylation in mammalian cells [3], CKII activation following serum stimulation is not in phase with the increase we observed in Cdk2pT39. CKII activity peaks within 30 minutes of serum activation and returns to basal levels within two hours [3].

Our data shows that Cdk2 forms a complex with Akt and is phosphorylated by Akt in vitro. Cdk2 reacts with an antibody that detects phosphorylated Akt products. This phosphorylation is absent in quiescent MCF-7 and increases rapidly upon mitogenic stimulation and is rapidly lost upon PI3K pathway inhibition. Of particular interest was the temporal correlation between this phosphorylation event, cyclin-Cdk2 binding the accumulation of CdkpT160 and Cdk2 activation.

The activation of Akt, as observed by phosphorylation at AktS473, preceded and was temporally linked with the phosphorylation at Cdk2T39. Upon mitogen stimulation of quiescent cells, T39 phosphorylation appears to precede both T160 phosphorylation of Cdk2 and is association with cyclin E. Drug induced PI3K inactivation caused a rapid loss of Cdk2T39 phosphorylation, that preceded the loss of Cdk2pT160 and disassembly of Cyclin E-Cdk2 complexes, despite no loss of cyclin E levels in the LY294002 treated cells.

Our in vitro assembly data and the immunoprecipitation data obtained from *CDK2P*$^{-/-}$P MEFs transfected with Cdk2wt, Cdk2P^{T39E}P and Cdk2P^{T39A}P also suggest that this phosphorylation event affects either the formation or stability of cyclin-Cdk complexes. The discordance between the *in vitro* data (which indicates that the rate of cyclin binding is faster, although the total binding reached is similar) and the cellular data (which indicates differences in steady state abundance of cyclin-Cdk complexes) may reflect the additional effect of Cdk activation upon cyclin stability in cells in vivo. It is possible that alterations in proteolytic degradation or expression of cyclin (an event frequently seen in

transformed cells) triggers an accumulation cyclins and therefore we were able to observe a greater amount of cyclin bound to Cdk2 in the Cdk2-null MEFs transfected with T39E.

In order to avoid any compensatory mechanisms that could arise in transformed mammalian cells and Cdk2 null MEFs, we used the yeast model system to determine if mutations affecting cdc28S46 had a cell cycle phenotype. Previous studies using in *S. cerevisiae* had demonstrated Cdc28S46 phosphorylation in vivo [26]. Mutation converting Cdc28S46 to alanine reduced cell volume and protein content, but a role for this site in cell cycle progression had not been defined. We observed a slight but highly reproducible shortening of the G_1 phase in the strains containing Cdc28S46E. This shortening of the G_1 phase did not give these cells a growth advantage, possibly due to triggering a morphogenesis checkpoint. Asynchronous yeast lysates, as well as lysates from time points collected after release from quiescence indicated that the cdc28S46E had a greater catalytic activity than Cdc28WT, as previously published[26]. The steady state levels of Cln2 rose earlier in the cdc28S46E strain, but reached lower peak levels despite higher peak cdc28 catalytic activity. This may reflect the effect of Cdc28/Cln2 kinase to promote Cln2 degradation. The in vitro and in vivo date presented support a model in which phosphorylation of Cdk2 at T39 and of Cdc28 at S46 may promote more rapid assembly with cyclins, and modulate the timing or stabilization of T160 phosphorylation. This novel regulation mechanism through which phosphorylation of G_1 Cdks, specifically but perhaps not limited to Cdk2, would influence the duration of G_1 phase.

Our current understanding of Cdk activation and cell cycle progression contains very little in terms of post translational modifications that alter the affinity of Cdks for cyclins. To date, the known phosphorylations events in Cdks alter the cyclin-Cdk catalytic activity. Across the cell cycle, the phsphorylations in Cdk2Y15 and Cdk2T160 increase through G_1 [5,8]. This may be due to the increase in activity of wee1 kinases as a response to mitogenic stimulation and the constant activity of CAK [6,30]. Although Cdk2 can be acted upon by CAK when in its monomeric form, the accumulation of Cdk2pT160 is a direct function of cyclin binding as cyclin association prevents dephosphorylation of the T-loop.

Fisher et. Al. have put forth a model whereby Cdk2 is phosphorylated at Cdk2T160 prior to cyclin binding [16]. The observation that cyclin-Cdk binding is required for nuclear import of the complex raises a problem with the model of Fisher et al. Since Cdk-activating kinases *in both budding and fission yeast* appear to localize to the nucleus, it would be difficult for the CAK complex to phosphorylate CKD2 complexes prior to its binding to cyclins, since the Cdk2 monomers appear to be largely cytoplasmic [11]. We envision Cdk2T39 phosphorylation serving as a trigger mechanism that facilitates cyclin-Cdk assembly and possibly also nuclear import. Previous work has shown that cytoplasmic mislocalization of active cyclin A-Cdk2 leads to apoptosis [9]. The PI3K pathway is a strong mitogenic and anti-apoptotic signal. Thus it is possible that constitutive activation of the PI3K pathway in cancers inhibits, or alters the rates of nuclear export versus import, of cyclin-Cdk complexes, thereby preventing apoptosis and driving the cells toward S-phase. This way, extracellular insults such as gamma irradiation would not cause a cell cycle checkpoint and possibly lead to genetic instability.

FUTURE DIRECTIONS

The findings of my thesis work suggest that activation of the PI3K pathway, and its downstream effector Akt, leads to the phosphorylation of Cdk2T39. This, in turn, causes Cdk2 to form complexes with its cyclin partners more rapidly and results in an active kinase that has a greater catalytic activity. In yeast cells, the phosphomimetic cdc28S46E has a shorter G_1-S phase transit time. There are several unresolved questions that arise from this work that warrant further investigation in the Slingerland lab. The following proposed experiments would extend my current line of investigation and further explore the mechanisms whereby the PI3K pathway regulates Cdk2 activity.

Inquiry 1: How Does Cdk2T39 Phosphorylation Affect Subcellular Cdk2 Localization?

The experiments above indicate that the phosphorylation at Cdk2T39 occurs prior to the increase in phosphorylation at Cdk2T160 as cells progress from G_0 to S phase. In addition, PI3K pathway inhibition caused a rapid loss of phosphorylation at Cdk2T39 which preceded the loss of the Cdk2T160 phosphorylation. This temporal link between the two sites, coupled with the shorter G_1 to S phase transit seen in yeast strains carrying a T39 phosphomimetic mutation in the Cdk2 homologue, cdc28, suggest that phosphorylation at the T39 site positively regulates the subsequent action of the Cdk activating kinase (CAK) on Cdk2. This may occur directly through a conformation effect on Cdk2. Alternately, the greater action of CAK on Cdk2 may be driven by a T39-dependent translocation of Cdk2 to into the nucleus.

The size of Cdk2 (34 kDA) and cyclins E and A (54 and 60 kDA, respectively) would permit them to translocate freely through the nuclear pores between nucleus and cytoplasm. However, the cyclin-Cdk2 complexes localize to the nucleus in late G_1 in parallel with their periodic catalytic activity, indicating that Cyclin-Cdk2 localization is actively regulated. CAK is predominantly nuclear localized throughout the cell cycle; cyclins E and A accumulate in the nucleus in late G_1 and S phases, while Cdk2 is both nuclear and cytoplasmic$P^{145}P$. Cdk2 substrates are mostly nuclear proteins. Chiefly among them are histone H1 and the retinoblastoma protein$P^{146}P$. Thus, if Cdk2 T39 phosphorylation enhanced the rate of nuclear import of Cdk2, or cyclin-Cdk2 complexes toward their sites of action in the nucleus, this could increase the amount of active cyclin-Cdk complex present in the cell.

Experiment 1: Does the Phosphomimetic Mutant cdc28S46E Show Enhanced Nuclear Localization Compared to Wild Type?U

We have created yeast strains that contain Cdc28wt, cdc28S46A and cdc28S46E. These strains could be synchronized in G_1 by alpha factor synchrony experiments and collected at different time points as they re-enter the cell cycle. The localization of Cdc28 and the mutant cdc28 proteins could be visualized by immunofluorescence. We would expect to see that the cdc28S46E mutant may accumulate in the nucleus earlier and or at higher levels than would be observed for Cdc28 and cdc28S46A.

Experiment 2. Does Cdk2pT39 Increase Binding to Importin-α?U

The nuclear import of cyclin E-Cdk2 (and of cyclin E alone) has been shown to require binding to the import proteins importin-α and importin-β (Moore et al., 1999). Cdk2 lacks a nuclear import signal. Its translocation in to the nucleus is in part mediated by its binding to cyclin E, because that latter contains a nuclear localization signal that mediates importin-α binding. One mechanism whereby T39 phosphorylation on Cdk2 may enhance nuclear localization could be via an enhanced interaction between cyclin E-Cdk2 complexes with the importin proteins. Thus, it would be of value to test if T39-phosphorylated Cdk2 (Cdk2pT39) may have a greater affinity or enhance the stability of association with the importins compared to non-phosphorylated Cdk2.

This could be tested by comparing mixtures of recombinant cyclin E with either Cdk2wt, Cdk2T39A or Cdk2T39E proteins already present in the lab in an in-vitro binding assay using commercially available, recombinant, GST-tagged importin-α. Alternatively, we could pre-treat Cdk2wt with Akt and then use it in binding assays with importin-α in the presence of cyclin E. The phosphorylation of Cdk2 at T39 may not only enhance cyclin E-Cdk2 complex association, but may also enhance to binding of the cyclin E-Cdk2 complex to importin-α. Controls in these experiments would include binding reactions of the different Cdk2wt, Cdk2T39A or Cdk2T39E proteins to the importin, in the absence of cyclin E and binding of the cyclin E to importin-α alone, in the absence of Cdk2. An increase in the steady state levels of importin-α to recombinant cyclin E-bound Cdk2T39E

or Akt pre-treated Cdk2wt, compared to Cdk2wt, untreated with Akt, would indicate a mechanism whereby cyclin E-Cdk2 complexes could indeed accumulate in the nucleus more readily.

Experiment 3: Effects of Cdk2pT39 on Nuclear Import *in vitro*.

We could assay the in vitro nuclear import of recombinant Cdk2-cyclin or Cdk2 alone into isolated nuclei as follows. Cells are briefly permeabilized by treating with digitonin which permits escape of cytosolic proteins, leaving behind "bare nuclei." These nuclei could then be reacted with recombinant Cdk2, recombinant importin-α/β, RanGDP and an ATP generating system$P^{147}P$. Following incubation for specific intervals, we would verify the extent of nuclear Cdk2 import by separating the nuclei from the supernatant via centrifugation, and assaying for imported Cdk2 protein by lysis of the nuclei followed by western blotting. Here too, we could use either recombinant Cdk2T39E or we could pre-treat recombinant Cdk2 wt with Akt and compare its rate of import to non-treated Cdk2, both in the presence and absence of recombinant cyclin E. We anticipate that the cyclinE-Cdk2 complex will be imported more rapidly when the Cdk2 is either pre-treated with Akt or the phosphomimetic variant is used. We anticipate that Cdk2 import will be affected by the T39 status only when the import assays are carried out in the presence of cyclin, since only the latter is capable of binding the importin-α/β machinery.

Significance: These assays may illuminate further the mechanisms through which the PI3K pathway promotes Cdk2 activation in higher eukaryotes.

Inquiry 2: Does Cdk2T39 Phosphorylation Make It a Better Substrate for CAK?

Full activation of Cdks requires T160 phosphorylation at the T-loop via CAK. Our data indicates that phosphorylation of Cdk2 at T39 precedes that on T160 in cells and induces a faster rate of cyclin-Cdk complex formation in vitro. Moreover, the phosphomimetic mutation of the yeast Cdk homologue at this site, cdc28S46E, has a higher catalytic activity than the Cdc28wt. In the case of Cdk1, CAK action requires prior cyclin binding. In contrast, Cdk2 is thought to be phosphorylated by CAK as a monomer, but cyclin

binding protects the T160 site from dephosphorylation. Since we have observed that the appearance of the Cdk2pT39 precedes that of Cdk2pT160 during G_0-S phase progression, this raises the possibility that T39 phosphorylation may condition the Cdk2 for action by CAK. T39 phosphorylation may not only promote more stable cyclin association, but may also modify the conformation of the Cdk2 monomer to permit more ready phosphorylation by CAK.

Experiment 1: Is the Steady State Binding of CAK and Cdk2 Altered by Mutations at T39?

Some kinases are known to form transient complexes with their substrates (Brazil, 2002). Thus, we would like to determine if the CAK-Cdk interaction would be altered by Cdk2T39 mutations. To do this, we could transiently transfect MCF-7 cells with HA tagged Cdk2 constructs and immunoprecipitate using an anti HA antibody. These precipitates could then be used to probe for one of the subunits of CAK (Cdk7, Mat A, Cyclin H). Changes in the steady state binding could be indicative of a greater affinity between CAK and Cdk2 or a greater stability of the complex, once formed.

Experiment 2: Does Cdk2T39E Serve as a Better *in vitro* CAK Substrate?U

This could be tested by performing a kinase assay using recombinant, active CAK expressed from bacculovirus in insect cells and recombinant Cdk2 as a substrate. Differences in the rate or amount of Cdk2pT160 product formed from Cdk2T39E, Cdk2T39A and Cdk2wt substrates would indicate either differences in CAK affinity for the substrate or efficiency of the reaction. Additionally, we could pre-treat Cdk2wt with Akt and test if the rate or extent of the action of CAK on Cdk2 was affected.

Cak1p is the *S cerevisieae* homologue of the human CAK that phosphorylates Cdc28 at T169 (the site homologous to T160 in human Cdk2). As an alternative strategy, one could immunoprecipitate Cdc28, cdc28S46E and cdc28S46A from alpha factor treated cells and compared these substrates in a Cak1p kinase assay. Long term treatment with alpha factor would abolish most of the T169 phosphorylated cellular Cdc28 such that the immunoprecipitated complexes could serve as substrate in a Cak1p kinase assay.

Significance: Determining if Cdk2T39 phosphorylation plays a role in CAK function would be instrumental in elucidating the mechanisms linking both T39 and T1260 phosphorylation events and how activation of the T39 phosphorylation event shortens the G_1 to S phase transit time

Inquiry 3: How Does Expression of the More Catalytically Active *cdc28S46E* protein Affect Cln2 Stability and Phosphorylation?

Our data using the budding yeast model system showed that cdc28S46E had a higher catalytic activity than Cdc28. Additionally, we repeatedly saw that the cdc28S46E strain had a lower steady state level of Cln2, even though the levels of Cdc28 and PGK1 (a cytoplasmic protein used as loading control, data not shown) were similar in both lysates. Since the Cln2 degradation is activated by its phosphorylation by Cln2-Cdc28P^{147}P this might explain the lower levels of Cln2 in the yeast expressing cdc28S46E. Thus, we would like to determine if the increased catalytic activity of the phosphomimetic mutant could be turning on a negative feedback loop and thereby decreasing the levels of Cln2 present in the cells.

Experiment 1: Does the Half-life of Cln Differ Between Cdc28 Strains?U

To test this, we would perform a cycloheximide chase and determine the half life of Cln2 in Cdc28, cdc28S46A and cdc28S46E strains. We could assay the loss of Cln2 protein by recovering lysates at intervals after cycloheximide treatment and immunoblotting the lysates for HA (a tag that was added to the *CLN2* gene). Alternatively, we could pulse label the cells by treating them with [P^{35}PS]-methionine, transfer to chase media containing cold methionine and then assay the decay of incorporated radioactivity in HA-Cln2 at intervals thereafter by HA-immunoprecipitation, resolution on SDS-PAGE and autoradiography of dried gels.

Experiment 2. Would Proteasome Inhibition Restore the Concentration of Cln2 Proteins to Similar Levels in Cdc28 and cdc28S46E?U

We postulate that the increases activity of cdc28S4E is triggering a negative feedback loop leading to degradation of its Cln2 partner. If this is indeed the case the treatment with MG132 should diminish the difference if Cln2 concentration between the two strains. We could treat either asynchronously growing cells, or cells that are 20 to 30 minutes into the cell cycle from an alpha factor release with a proteosomal inhibitor (MG132). The proteosomal inhibitor would inhibit the degradation of Cln2 and therefore we could observe if indeed the different levels of Cln2 are due to increase degradation.

Significance: It is well established that for several G_1 cyclins, including both cyclin E and Cln2, degradation is triggered by Cdk-mediated cyclin phosphorylation. The finding that cdc28S46E has a lower Cln2 concentration than that in the Cdc28wt strain, provides an in vivo validation of our data showing that cdc28S46E has greater catalytic activity.

Inquiry 4: Do Changes in T39 Phosphorylation Have Different Consequences in Cancer-derived Versus Normal Somatic Cells of Finite Lifespan?

In several cancers, it has been shown that G_1 cyclins, cyclin D1 and cyclin E are stabilized through mechanisms that are not entirely clear. Moreover, Cdk2 is often overactivated in cancers by constitutive receptor tyrosine kinase activation (such as Met, Her2 or EGFR), activating mutations of the catalytic component of PI3K, *PIK3CA*, or of PI3K effectors including *Akt*. Cancers may select for cyclin overexpression or for greater cyclin stability to allow the cancer cell to overcome the negative feedback look that occurs after a threshold of Cdk activity is achieved. This would permit the neoplastic cells to maintain an abnormally high mitogenic signal and enforce cells accelerated G_1 cell cycle transit.

One of the biggest confounding factors in the study of Cdk2 in mammalian cells is the use of either immortalized or cancer-derived transformed cells to study a phenotype that, in yeast, is relatively modest. Deregulation of cell cycle controls and increased G_1-S phase transit is a universal hallmark of cancer cells. Transformed cancer-derived cell cultures have undergone a selection yielding a very robust cell cycle. Cdk2 activation is frequently observed in cancer cells$P^{148}P$, raising the possibility that these lines are “oncogene-addicted” to activated Cdk2. This possibility is supported by the observation that in

cultured cells derived from glioblastomas and osteosarcomas, inhibition of Cdk2 prevents proliferation [33] [31], while cell cycle proliferation in embryogenesis is not perturbed in *CDK2* $^{/-}$ mice and these animals do not show alterations in their brains or connective tissues [20].

Given the frequent deregulation of the PI3K pathway in human cancers, one might expect that the phenotype of a Cdk2T39E may be lost in a cancer cell line. The following are proposed to investigate the consequences of T39 phosphorylation in malignant versus normal finite lifespan cell types.

Experiment 1: Is There a Difference in the Timing of Cdk2 T39 Phosphorylation and Steady State Cdk2-Akt Binding Between Transformed and Primary Cells?

In a first set of experiments, I propose to compare the timing of Cdk2T39 phosphorylation during G_1 to S phase in a series of cancer-derived cell lines (breast and lung) and normal finite lifespan epithelial cells from breast and lung tissues. I will chose cancer lines that can be synchronized in quiescence by growth factor or serum deprivation. These lines will be compared with regard to the kinetics of the T39 phosphorylation of Cdk2, the duration of G_0-S phase cell cycle progression, and the timing of cyclin-Cdk2 binding and Cdk2 activation. I would anticipate that many of the malignant lines would show a shorter G_1-to S duration and more rapid onset of T39 phosphorylation on Cdk2, cyclin-Cdk2 complex formation and activation, and that this would be directly proportional to their degree of oncogenic Akt activity compared to normal epithelial cells so the same tissue origin.

I would also test if extent and timing of the steady state binding between Akt and Cdk2 differs between primary cells and transformed cells. As Akt is more active in many malignantly transformed lines, I expect the steady state binding between these two molecules would be lower in primary cells.

Experiment 2. Does Replacement of Cdk2 With Cdk2T39E Have Different Consequences in a Malignant Line Compared to Finite Lifespan Epithelial Cells?

Finally, it would be of interest to compare the consequences on G_1-S transit time when cellular Cdk2 is replaced by homologous recombination with Cdk2T39E in somatic cells of malignant origin versus finite lifespan epithelial cells. For this, one would replace the endogenous *CDK2* gene sequence with a *CDK2T39E* encoding gene. The sister lines so derived would be compared for the effects on G_1-to-S phase timing, Cdk2-Akt complex formation, Cdk2-cyclin-binding and Cdk2 activation. One would expect that in the transformed lines, that are already driven by a constitutively activated PI34K pathway, the expression of Cdk2T39E would have little effect. In contrast, replacement of cellular Cdk2 with the Cdk2T39E would shorten the G_1 transit time in normal finite lifespan epithelial cells, by increasing cyclin-Cdk2 complex formation and Cdk2T160 phosphorylation.

Significance: In the context of cancer cell lines, in which PI3K is already oncogenically activated or in which Cdk2 is already activated by loss of the Cdk inhibitors p21 or p27, or by *CDK2* gene amplification, the effects of a phosphomimetic Cdk2T39E mutation, or indeed loss of potential to phosphorylated T39 in a non-phosphorylated Cdk2T39A may not be readily apparent. A phosphomimetic mutation at Cdk2T39 that has a modest effect to facilitate cyclin-Cdk2 complexing and/or T160 phosphorylation in normal cells may not be readily apparent in cancer cells that are progressing through cell cycle at already maximal speed.

Experiment 3 What Would Happen to Cdk2 Upon Transient Transfection of Primary Cells With Constitutively Activated myr-Akt or AktUPU^{DD}UP?

By transiently transfecting a vector encoding a constitutive active Akt into primary cells I could determine if there are any differences in Cdk2T39 phosphorylation, Cdk2 subcellular localization and activation. I expect that transiently transfected cells would have an increase in Cdk2pT39, increased nuclear Cdk2, greater cyclin-Cdk2 steady state levels and higher catalytic activity.

Significance: Demonstrating that the timing of Cdk2T39 phosphorylation and Cdk2 subcellular localization is affected by constitutive activation of Akt would provide further

support for the notion that oncogenic deregulations in the PI3K pathway can push transformed cells prematurely through G_1 S of the cell cycle in response to, and indeed independently of mitogenic signals. This, in turn may lead to chromosomal aberrations which could promote neoplastic tumor progression.

KEY RESEARCH ACCOMPLISHMENTS:

- Obtained recombinantly expressed Cyclin E from Dr Caleb McDonald, from the Farooq lab at the University of Miami.
- Optimized protocol for recombinant protein binding assay.
- Determined the time needed for the majority of CDK2 to be phosphorylated by AKT in vitro
- Optimized the immunoprecipitation of kinase assay protocol to determine cdc28 catalytic activity throughout the cell cycle.

REPORTABLE OUTCOMES:

- Observed an increased rate of cyclin-CDK2 complex formation when using the CDK2T39E isoform
- Increased rate of formation was not followed by an increase in steady state binding as all CDK2 isoforms reached the same saturation rate.
- Pre-treatment of CDK2wt with AKT in an in vitro kinase assay increases the rate of cyclin-CDK2 complex formation compared to mock – or untreated CDK2.
- In yeast, the CDK2T39E homologue cdc28S46E has a higher catalytic activity in vitro.

- Cdc28S46E showed a higher catalytic activity in all timepoints assayed after cells were released from alpha factor arrest.
- Data gathered during the grant period was submitted and accepted for my thesis dissertation.
- The work presented in this grant is being prepared for publicantion. Manuscript is set to be sent off within the next two months.

APPENDIX 1 – FIGURES

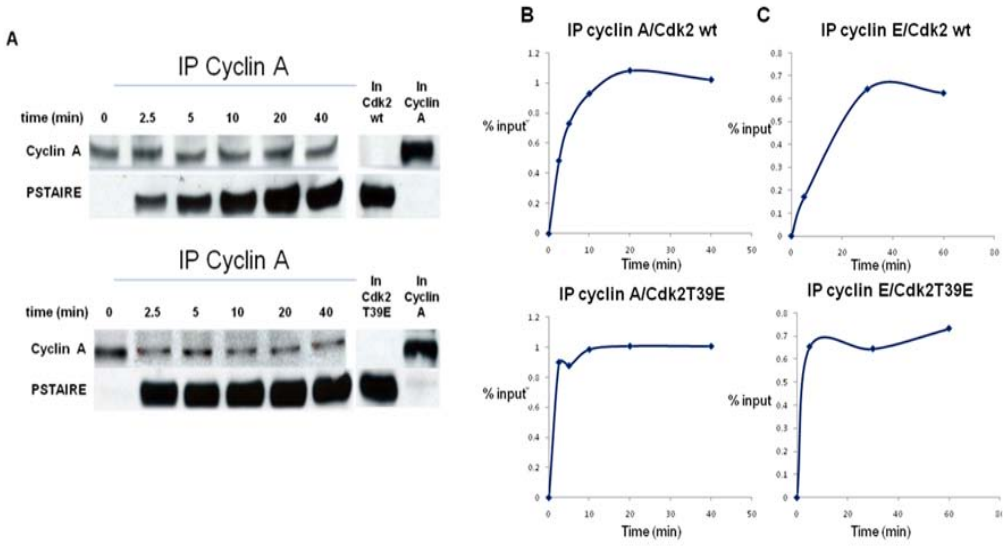

Fig 1. Phosphorylation of Cdk2 by Akt facilitates cyclin-Cdk2 assembly *in vitro* 10nM of purified His-tagged human Cdk2 and Cdk2T39E were reacted with 20 nM of purified recombinant human cyclin A or E. Levels of Cdk2-cyclin complexes formed were detected by cyclin immunoprecipitation followed by immunoblotting for Cdk2 using anti-PSTAIRE antibody. **(A)** Cyclin A- bound proteins shown in immunoblots with input into the binding reaction shown on the right. **(B)** Densitometric analysis of Cdk2 bound to cyclin A from IP-blots shown on left in (A) is graphed over time as a % of Cdk2 input into the reaction. **(C)** Densitometric analysis of Cdk2 bound to cyclin E is graphed as a % of the Cdk2 input into the reaction as in (B).

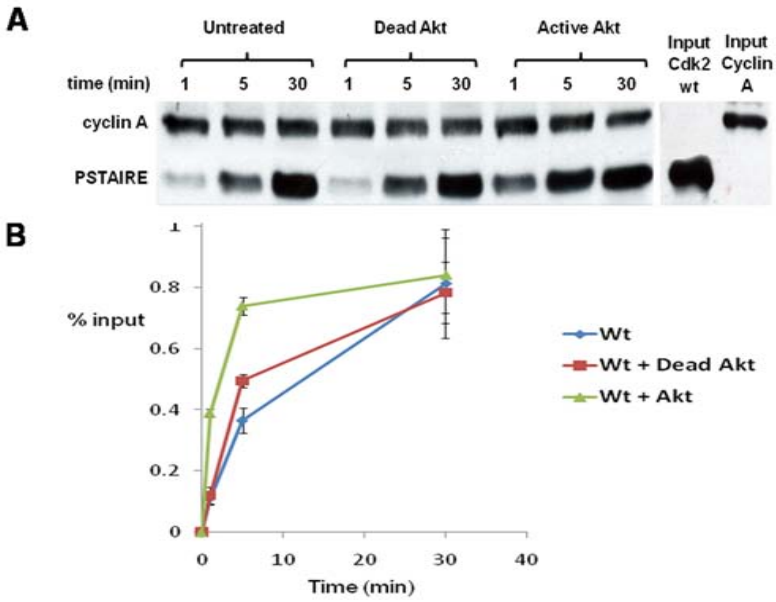

Fig 2. Phosphorylation of Cdk2 by Akt facilitates cyclin-Cdk2 assembly *in vitro* Purified His-tagged human Cdk2 was reacted with either a Catalytically active or inactive Akt under kinase conditions. Pretreated Cdk2 (50 nM) was then incubated with 100 nM of purified recombinant human cyclin A for the indicated times at room temp. Cdk2-cyclin binding was assayed by cyclin immunoprecipitation followed by immunoblotting for Cdk2. **(A)** Immunoblot of binding assay shows that phosphorylation of Cdk2 by Akt increases the rate of cyclin binding. **(B)** Densitometric analysis of binding assay.

Cdc28	MATa *CDC28::NAT*[WT *CDC28* in pRS415] *ade1 leu2-3, 112 his2 trp1-1 ura3Δns bar1Δ cln2::CLN2-3xHA::URA3* pRS415-*CDC-28*
Cdc28-S46A	MATa *CDC28::NAT*[*CDC28-ala* in pRS415] *ade1 leu2-3, 112 his2 trp1-1 ura3Δns bar1Δ cln2::CLN2-3xHA::URA3* pRS415-*cdc28-S46A*
Cdc28-S46E	MATa *CDC28::NAT*[*CDC28-glu* in pRS415] *ade1 leu2-3, 112 his2 trp1-1 ura3Δns bar1Δ cln2::CLN2-3xHA::URA3* pRS415-*cdc28-S46E*

Table 1. The CWY626 yeast strain was used as the parental strain for derivation of the Cdc28 genotypes used in the present study

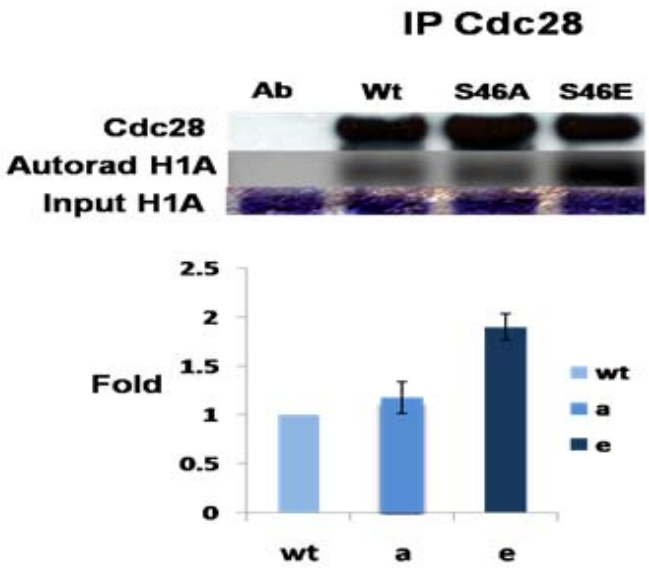

Figure 3. **cdc28-S46E has higher kinase activity than Cdc28.** Histone H1 kinase activity of stains bearing Cdc28, cdc28-S46A and cdc28-S46E. Exponentially growing cells were lysed and Cdc28, cdc28-S46A and cdc28-S46E mutants were precipitated from equal amounts of cell lysate using an anti-Cdc28 antibody as described in Materials and methods and assayed for Histone H1 kinase activity as described. **(A)** Immunoblotting of the precipitates used to determine Cdc28 kinase activity shows that an equal amount of Cdc28 was present in all reactions (top band), radioactivity in Histone H1 (middle band), and Coumassie stain of H1 substrate used in the reaction (bottom band) **(B)** Densitometric analysis of bands obtained from autoradiograph. Bars reflect the average of three individual experiments.

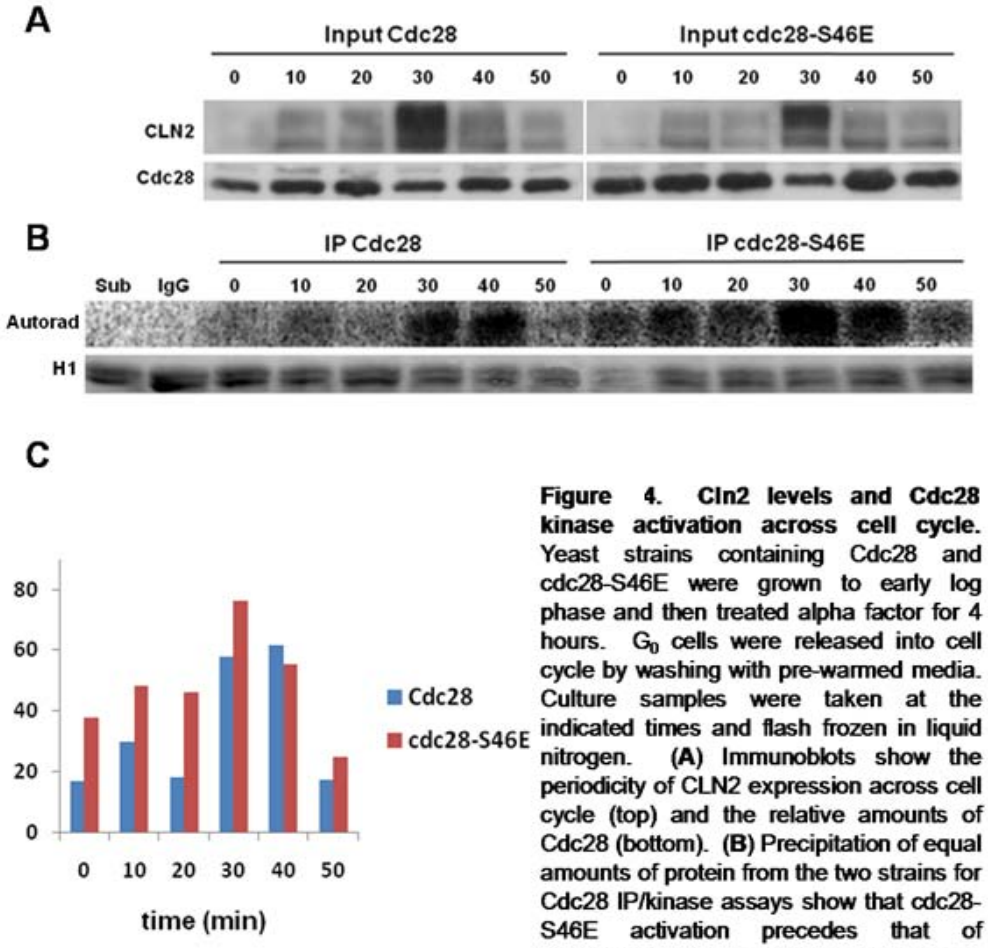

Figure 4. Cln2 levels and Cdc28 kinase activation across cell cycle. Yeast strains containing Cdc28 and cdc28-S46E were grown to early log phase and then treated alpha factor for 4 hours. G_0 cells were released into cell cycle by washing with pre-warmed media. Culture samples were taken at the indicated times and flash frozen in liquid nitrogen. **(A)** Immunoblots show the periodicity of CLN2 expression across cell cycle (top) and the relative amounts of Cdc28 (bottom). **(B)** Precipitation of equal amounts of protein from the two strains for Cdc28 IP/kinase assays show that cdc28-S46E activation precedes that of Cdc28WT and has reaches higher peak kinase activity during G1 progression. **(C)** Quantitation of radioactivity in H1 kinase substrate from kinase assays of Cdc28WT versus cdc28S46E.

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APPENDIX 3 – THESIS DISSERTATION.

UNIVERSITY OF MIAMI

PHOSPHORYLATION OF CDK2 AT THREONINE 39 BY AKT FACILITATES CYCLIN-CDK2 ACTIVATION

By

Thiago Bezerra Gaspar Carvalho da Silva

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

December 2010

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

PHOSPHORYLATION OF CDK2 AT THREONINE 39 BY AKT FACILITATES CYCLIN-CDK2 ACTIVATION

Thiago B.G.C.da Silva

Approved:

_____ Joyce Slingerland, M.D., Ph.D. Professor of Biology	_____ Terri A. Scandura, Ph.D. Dean of the Graduate School
_____ Thomas K. Harris, Ph.D. Associate Professor of Biochemistry and Molecular Biology	_____ Kerry Burnstein, Ph.D. Professor of Pharmacology
_____ Terace Fletcher, Ph.D. Kerry Burnstein, Ph.D. Associate Professor of Biochemistry and Molecular Biology	_____ Gennaro D'Urso Professor of Pharmacology

B.G.C. da Silva, Thiago (Ph.D., Biochemistry and Molecular Biology)
Phosphorylation of CDK2 at threonine 39 (December 2010)
By AKT facilitates cyclin-CDK2 activation

Abstract of a dissertation at the University of Miami.

Dissertation supervised by Professor Joyce Slingerland.
No. of pages in text. (86)

A model of individual differences in zoned residential land sales was evaluated using analytic techniques pertinent to critically refining realty science principles to ensure compliance with the coming new millennium market demands for land on which to build homes, dwellings, and residential units of various types heretofore beyond the powers of comprehension afforded the current real estate buyer. Variables including surface gradience factors, foundational permanence factors, garage conversion and sun porch additions were examined in their relation to outcome symptom level. It was predicted that environmental and temperamental variables would relate to process variables, which would in turn affect individual differences in outcome, based on geographical situation and civil locality of the parcel for sale. It was concluded that while the results of the medieval apprenticeship system in other parts of academe speak for themselves, often at great length, this method may not be the ideal one for fields involving contact with the outside world. A recommendation for further study is enclosed.

DEDICATION

I dedicate my dissertation work to son Dylan and to my family. A special feeling of gratitude to my loving parents, Kleber and Maria, who lead by example and always had words of encouragement. My brothers Kleber Jr. and Felipe, who despite the distance never left my side. Your love has served as my source of encouragement.

I dedicate this work and give special thanks to my uncle Klezer, who lived with me for several months during the beginning of my PhD. You brought light into what was a dark future.

I also dedicate this dissertation to my extended family who have supported me throughout the process. I will always appreciate all they have done. A special thanks to Annemarie and Mark Weems – my American parents – for their continued support and guidance.

I dedicate this work to Donna da Silva, who has been by my side despite our separation. Thank you and you will always be loved.

ACKNOWLEGEMENT

I wish to thank my committee members who were more than generous with their expertise and precious time. A special thanks to my mentor Dr. Joyce Slingerland, for her countless hours of guidance, reflection and encouragement. Thank you Dr. T. K. Harris, Dr. Terace Fletcher and Dr. Kerry Burnstein for agreeing to serve on my committee.

Special thanks to Dr.Tan for his friendship and technical expertise and to Dr. Myers for helping me broaden my scientific horizon.

I would like to acknowledge and thank the department of Biochemistry and Molecular Biology for accepting me into the program despite a less than stellar undergraduate GPA. I hope I lived up to your expectations.

Finally I would like to thank my good friends Ilene, Ken, Georgeta, Heath, Alex, Maria, and Steve whose excitement and willingness to provide feedback made the completion of this research an enjoyable experience.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

Chapter 1

INTRODUCTION

The Cell Division Cycle

The cell cycle is composed of a series of highly regulated steps that result in the division of a single cell into two. The transmission of genetic information requires the stringent control and faithful completion of events that are critical for processes such as differentiation, proliferation, growth and overall cellular homeostasis. There are four main phases in the cell cycle: G_1, S, G_2 and M phase. Upon mitogenic stimulation, cells that were not actively dividing progress from a quiescent state (G_0) into the initial phase, G_1, during which a threshold size is reached and specific proteins are activated. The commitment to enter DNA synthesis occurs at this "restriction point" or "start point" late in G_1 phase. In the event of DNA damage, a cell can undergo repair and delay passage through the restriction point or decide to undergo programmed cell death, or apoptosis, in the G_1 phase. After passing the restriction point in G1, mitogenic signals are no longer needed as the cell is committed to complete one round of cellular division. Beyond the restriction point, the cell enters the S-phase. During S-phase, cells replicate their DNA thus doubling their genetic load. With the completion of DNA replication, cells progress through a short growth phase,

Gap2 (G_2). This phase employs several regulatory mechanisms to ensure that the genome has been replicated once and only once. In the final stage, mitosis (M phase), the nuclear envelope breaks down, the chromosomes condense, and are equally divided between the two identical daughter cells that in turn, either initiate a new cycle or return to the quiescent state.

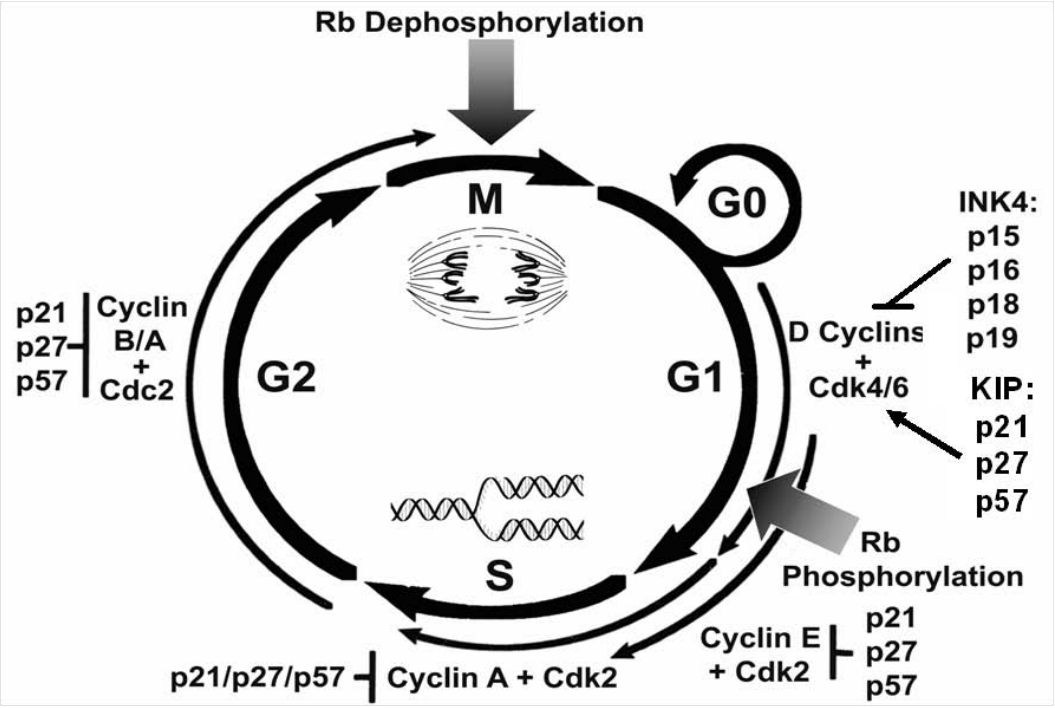

Figure 1. The cell cycle. Activation of D-, E- and A-type cyclin-Cdks promotes G_1-to-S phase progression. The INK4 family members inhibit cyclin D-bound Cdks. The KIPs (p21, p27 and p57) inhibit Cdk2 and facilitate assembly and activation of D-type cyclin-Cdks.

The First Cyclins and CDKs

At its core, the cell cycle is regulated by the periodic activity of heterodimeric protein kinase complexes composed

of cyclins and cyclin dependent kinases (CDKs). The cyclinc activation of these cyclin/CDK complexes is in part due to the timed expression and degradation of the cyclin proteins. Cyclin/CDK complexes play a central role in the control of the cell cycle by phosphorylating target proteins. The phosphorylation of specific substrates is critical in the operation of the ordered sequence of events leading to DNA replication and chromosomal segregation. The synthesis and accumulation of new cyclins is required for the formation of CDK/cyclin complexes at distinct points in the cell cycle. Rapid degradation and new cyclin synthesis throughout the cycle ensures proper progression into the next phase of the cell cycle and ensures that the cell cycle will not regress.

The first observations of cyclin proteins were made in cleaving eggs of marine invertebrates. Cyclins were so named because of their pronounced synthesis during interphase followed by abrupt destruction at the metaphase/anaphase transition [1-3]. Later, experiments using *Xenopus Laevis* oocytes showed that these proteins played a role in cell cycle progression. Microinjecting the cytoplasmic content from eggs arrested in metaphase of meiosis II into G_2-arrested oocytes lead them into meiotic entry, even in the absence of protein translation [4, 5]. This M-phase-promoting factor (also referred to as maturation promoting factor), or

MPF, was subsequently found in other eukaryotic organisms [6] and it was later determined to be a heterodimeric protein kinase complex composed of two subunits: a mitotic cyclin and a cyclin-dependent kinase (CDK) [7, 8]. Cyclins homologous to those found in *X. laevis* were later discovered in the yeast *S. pombe* and *S. cerevisae.* Three genes in budding yeast, CLN1, CLN2, and CLN3 (formerly DAF1), showed homology to mitotic cyclins yet functioned in the G_1 phase and were henceforth named the G_1 cyclins [9-12]. In the yeast *S. pombe* and *S. cerevisae,* cyclins are found associated with serine/threonine protein kinases called Cell-division-cycle, or Cdc, which are homologous to CDK found in *X. laevis*. In *S. pombe,* the protein kinase subunit of MPF is encoded by CDC2 [13], while in *S. cerevisae,* the protein kinase subunit is called Cdc28 [14]. The *S. pombe* Cdc2 protein shows functional homology to the Cdc28 protein of *S. cerevisae* and the two kinases perform similar functions in the different yeast species [9, 14]. Coordinated interaction between these protein kinase regulatory subunits and the cyclins is necessary for the proper progression through the cell cycle.

Cyclins

Eukaryotic cells have developed a number of functionally different cyclins that are specific to each phase of the cell

cycle. The cyclin family is comprised of many different proteins. These cyclins share a conserved homologous region comprised of about 100 amino acids, termed the "cyclin box" [15], which allows them to interact and bind CDKs. The family of cyclins encompasses a variety of proteins including cyclins A, B, C, D, E, F, G, H, up to cyclin T. Of these cyclins, only four, A, B, D and E, are directly involved in cell cycle progression and transcription, whereas the remaining cyclins have indirect roles in the cell cycle. Many cyclins involved in the advancement of the cell cycle contain multiple family members that contribute to their overall function. The cyclins known as the "G_1 cyclins," cyclin D and cyclin E are comprised of D_1, D_2, and D_3, and E_1 and E_2, respectively. These cyclins were discovered for their ability to complement G_1 cyclin deletions in *Saccharomyces cerevisiae* [16-18], Cyclin B has three family members B_1, B_2 and B_3 which were first discovered in *Xenopus* egg extract [19]. The A-type cyclins contain two family members, A_1 and A_2, which were discovered for their close similarity to the B-type cyclins [3, 20]. None of these cyclins can exert kinase activity until they bind a cyclin-dependent kinase. Cyclin levels are tightly regulated across the cell cycle by periodic transcription and timed proteolysis. Cyclin E mRNA expression increases in mid G1 and cyclin E protein levels

rise sharply at the G1-to-S transition [21]. Cyclin A expression is induced in S phase through M phase. The ubiquitin-mediated degradation of cyclin E is activated through phosphorylation by its associated CDK at S384 [22-25]. Of particular interest to the present thesis work is that CDK2 activity is implicated in both Cyclin E gene expression and its protein degradation. Cyclin A is also phosphorylated at S154 by its bound CDK, although this phosphorylation is not required for its ubiquitin-mediated degradation [26].

Cyclin Dependent Kinases

The CDK family is made up of 20 different proteins, 11 of which are true cyclin-dependent kinases (CDK1-11) [27]. CDKs are broad specificity, proline-directed serine/threonine kinases with a consensus recognition sequence of: Ser/Thr-Pro-X-Lys/Arg [28]. CDK 1 (also known as Cdc2) and CDK2, CDK4, and CDK6 are necessary for cell cycle progression [29]. One additional family member, CDK7, regulates the cell cycle by operating as a key component of the CDK-activating complex (CAK) [30]. The role of CDKs within the cell cycle is to phosphorylate an array of distinct proteins. These substrates are phosphorylated on serine or threonine residues in a sequence specific manner that is recognized

by the active site of the CDK [31]. Although the catalytic site of the cyclin-CDK heterodimer lies exclusively within the CDK, the substrate specificity is determined by the combination of cyclin-CDK complex [32]. In general, the levels of CDK protein are constant and in excess compared to cyclin levels, thus the formation of cyclin-CDK complexes has been thought to be predicated largely on the expression of cyclins[33]. The work of this thesis provides new data that suggests that the periodic accumulation of cyclin-CDK complexes may not be strictly predicated by the abundance of cyclin alone, but rather may be affected by a timed phosphorylation event on the CDK.

Basic Framework of the Eukaryotic Cell Cycle

As with more primitive forms of life, the activity of mammalian CDKs requires the binding of cyclins. Cyclin expression, in turn, is tightly regulated according to the phases of the cell cycle. In early G1, activation of the mitogenic pathways including Ras-Raf-MAPK causes the transcription of D-type cyclins [34, 35], which bind and activate CDK4 and CDK6 [33] (Fig 1). The presence of growth factors increases D-type cyclin levels throughout early G1[36]. Cyclin D-CDK4/6 complexes phosphorylate and thereby partially inhibit the retinoblastoma protein (pRb) which

allows for activation of E2F transcription factors required for transcription of cyclins E and A that act a little later in G1 progression [37, 38]. Cyclin E is expressed in mid to late G1 phase [39]. Cyclin E binds and activates CDK2. Cyclin E/CDK2 phosphorylates different sites on pRb than do cyclinD-CDK4/6 complexes and the collaboration of both D-CDK4/CDK6 and E-CDK2 type complexes is required for complete inactivation of Rb [40]. Thus cyclin E-CDK2 creates a positive feedback loop through which more cyclin E is expressed. Cyclin E-CDK2 activity drives the cell into S phase and regulates expression of gene products needed for initiation of DNA replication [21]. Cyclin E-CDK2 can also phosphorylate Cyclin-CDK inhibitors and paradoxically it also mediates the targeted degradation of cyclin E itself (see below). Cyclin A is expressed at the G1/S phase boundary and its CDK-mediated activity drives the cell into mitosis. Cyclin A accomplishes this by forming complexes with both CDK2 and to a lesser extent with CDK1 (cdc2) [41, 42]. Cyclin B1 is expressed late in S phase; binds to CDK1 and is responsible for driving the cell into mitosis [43]. The activity of Cyclin Dependent Kinases can also be regulated through post-translational modifications and through binding to inhibitory subunits (see below)

The CDK Inhibitors: INK and KIP Families

There are two main classes of CDK inhibitors in mammalian cells: Inhibitor of CDK4 (INK4) and CDK Inhibitor Protein (CIP/KIP). The INK4 family includes p15 (INK4b), p16 (INK4a), p18 (INK4c), and p19 (INK4d) [44-47]. These INK4 members share four tandem ankyrin sequence repeats and target CDK4 and CDK6, the catalytic subunits of cyclin D, but not CDK2. They are believed to cause a G_1 arrest by competitively binding to CDK4/6, thereby preventing cyclin D from binding and forming a catalytically active complex [48].

The CIP/KIP family includes p21 (CIP1/WAF1), p27 (KIP1), and p57 (KIP2). These inhibitors share a homologous CDK inhibitory domain in the N-terminus region and a nuclear localization signal at the C-terminus end. They function by binding to cyclin-cdk complexes, including cyclin A/cdk2, and cyclin E/cdk2, thereby inhibiting kinase activity [49-52]. Interestingly, the CIP/KIP family of inhibitors may function as an assembly factor or nuclear targeting factor to Cyclin D/cdk complexes. Several studies have shown that inhibition of cyclin D/cdk complexes leads to an increase in unbound p21 and p27, which results in increased inhibition of cyclin E and growth arrest [53-55].

Regulation of Cyclin-CDK Activities by Phosphorylation

Cyclin/CDK complex activation is also subjected to post-translational modification by phosphorylation. CDKs contain activating and inhibitory phosphorylation sites. These have been thoroughly studied in yeast and human homologues. Binding of cyclins to CDKs yields a partially active complex. Full activation is achieved through dephosphorylation of inhibitory sites [56] and by phosphorylation of a site located on the T-loop (described below). This site is theonine (Thr) 172 for CDKs4 and 6, Thr 160 for CDK2 and Thr 161 in CDK1 [57-59]. This phosphorylation is carried out by an enzyme called CDK activating kinase (CAK). The mammalian CAK is a trimer composed of p40M015 (CDK7), cyclin H and the assembly factor, MAT1 [30, 60, 61]. Thr-160 phosphorylation generates a downward electrophoretic shift in CDK2. The timing of T-loop phosphorylation was, at first, thought to occur after cyclin binding. This idea was based on studies using homologous CDK in the fission yeast *S pombe.* In *S.pombe,* the CAK homologues (Mcs6, and Csk1) act on Cdc2 only once it has bound to the cyclin [62] and early evidence in mammalian cells suggested that cyclin binding was also needed for CAK phosphorylation of mitotic cyclin-CDK1 complexes [56]. Interestingly, data from studies using the budding yeast *S.Cerevisiae* challenged that

notion. In *S. cerevisiae*, the CAK homologue, Cak1p, can phosphorylate the Cdc28 in the absence of cyclin [63]. In humans, recent data suggest that the mechanism whereby CAK function differs for different substrate CDKs. This work provided further data supporting the model in which T-loop phosphorylation of CDK1 occurs only after cyclin is bound [64]. In contrast, it was proposed that for CDK2, CAK appears to phosphorylate CDK2 prior to cyclin binding, but cyclin binding serves to stabilize the T160 phosphorylation by preventing the rapid dephosphorylation of the T-Loop site by PP2A phosphatases [64]. Additionally, mammalian CAK is active throughout the cell cycle. Thus, the prevailing model holds that the phosphorylation of CDK2T160 occurs continuously across the cell cycle and that cyclin binding only stabilizes this phosphorylation and protects Thr-160-phosphorylated CDK2 from dephosphorylation by the CDK-associated phosphatase (KAP) and protein phosphatase 2a [65]. These two mechanisms have been deemed “cyclin first” and “CAK first” for CDK1 and CDK2, respectively. When first published in 2008, this data dramatically altered the model that had prevailed since the early 90s that held that monomeric CDKs were poor CAK substrates and that catalytic action of CAK was enhanced by cyclin binding.

CDKs contain two inhibitory phosphorylation sites. These sites reside on the N-terminus of the CDK, specifically on Tyr. 15 and Thr. 14 of CDK2 and CDK1, and on Tyr 17. of CDKs 4 and 6. These inhibitory sites are targeted by the Wee1 and Myt1 kinases. Phosphorylation of the inhibitory sites results in the loss of activity of the Cyclin/CDK complex – even if it is phosphorylated in its T-loop [66]. These phosphorylation events do not cause a change in electrophoretic mobility of the CDK.

The kinases that regulate CDKs are themselves regulated. Although CAK function seems to be constant throughout the cell cycle, cells that are deprived of growth factors do experience a loss of CAK function [67-69]. The activities of Wee1 and Myt1 are regulated via post-translational modification and changes in subcellular localization in a cell cycle-dependent manner [70]. These inhibitory phosphorylations are removed by the action of the Cdc25 family of phosphatases [71]. Here again, the activity of cyclin-CDK complexes causes a positive feedback loop, where activation of Cyclin E/CDK2 causes Cdc25A to be activated via Cyclin E/CDK2 phosphorylation [72].

CDK Conformational Changes Accompany Cyclin/CDK Asembly

The elucidation of CDK activation was assisted by structure studies involving the interaction of CDK2 and cyclins A and E [73, 74]. As with all protein kinases, CDKs have a tertiary structure containing a small amino-terminal or N-lobe rich in β-sheets and a larger helix-rich carboxy-terminal or C-lobe [73]. An ATP molecule fits tightly in a cleft between the two lobes in such a way that orients the phosphates outward, toward the mouth of the cleft. Substrates bind at the front of the cleft allowing substrate interaction primarily with the surface of the carboxy-terminal lobe. A catalytic transfer occurs among nearby residues setting off a transfer of the terminal γ-phosphate of ATP to hydroxyl oxygen in the protein substrate [73, 75]. There are key conformational changes that must take place so that CDK2 can be converted into a catalytically active form (Fig 2). First, the T-loop - a large flexible loop that contains a phosphorylation site for CAK and several residues which block substrate binding to the active site - must move away from the active site and thereby allow entry of ATP and substrate into the catalytic cleft. Second, the PSTAIRE helix must move so that in can allow for critical interactions between the cyclins and CDK. Two alpha helices, the PSTAIRE helix and the L12 helix, are important contributors for the control of CDK activity. Upon cyclin binding, the PSTAIRE helix is twisted and pushed up against

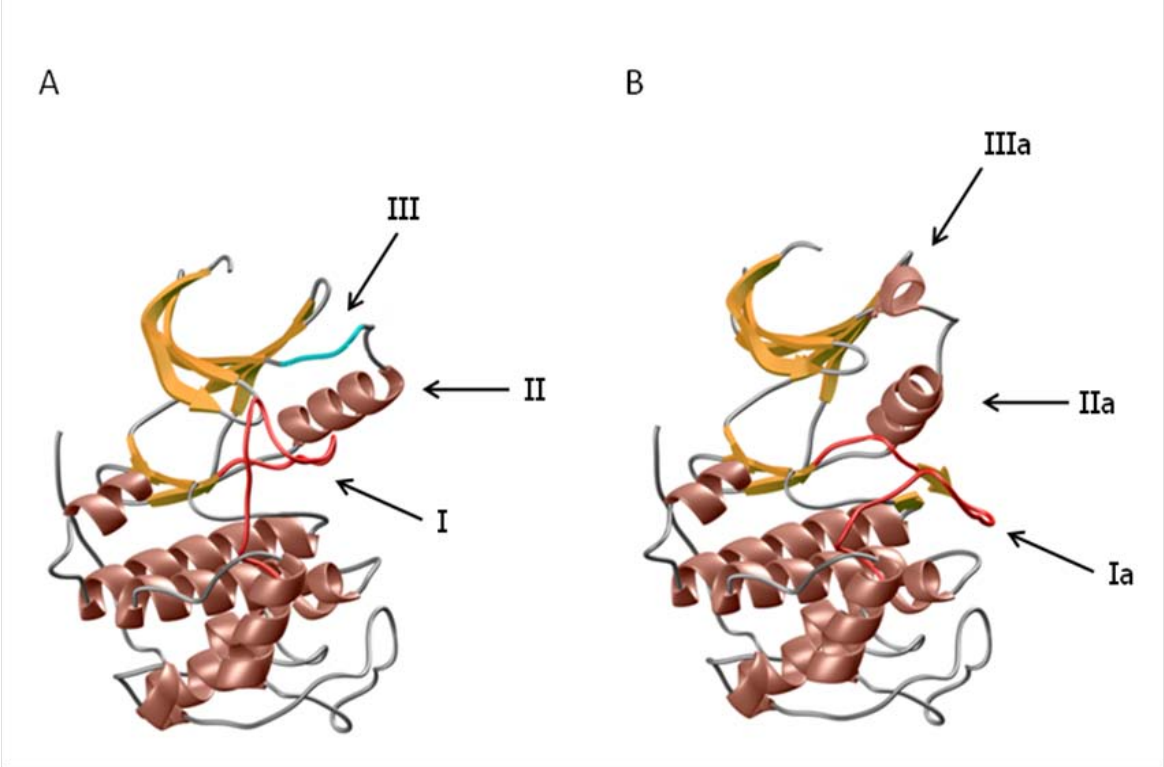

Figure 2: Crystal structure of Cdk2. Conformational changes between unbound (A) and cyclin E bound (B) Cdk2. Structures determined through crystallography. Blue area indicates a region that was not resolved in crystal data. Notice that cyclin binding causes the t-loop (I, red) to move out of the catalytic cleft (Ia), the PSTAIRE (II) helix to move 90° (IIA) and the formation of the 40s loop (from III to IIIa). This crystal structure of the bound cyclin is not shown. Phase data from Honda et. Al., 2005.

the L12 helix. This movement allows for the proper orientation of Glu51 - which is part of the catalytic triad responsible for ATP phosphate orientation and coordination of the activating magnesium ion – toward the formation of an ion bridge with Lys33 (Lys33, Glu51 and Asp 145 form the catalytic triad) [76]. Studies of the crystal structure of

CDK2 bound to cyclins E and A have found that both cyclins interact with CDK2 primarily through the PSTAIRE helix and that no major conformational changes occur in the cyclins upon binding [74].

Genetic Studies of Cyclins and CDKs

For many years it was believed that proper cell cycle progression through the G1 phase required strict timing in the expression and degradation of the known cyclins required for the sequential activation of specific CDKs. Recent information from gene-targeted mouse models for various cyclins and CDKs have caused some of the generally accepted concepts of the cell cycle to be revised (Table 1). It is now becoming clear that the linear input of different cyclin-CDK complexes on G1 progression is an oversimplification. There appears to be significant functional redundancy between different G1-S phase cyclin–CDK complexes during embryogenesis, such that engineered loss of one cyclin in the developing mouse embryo is complemented by action of another and the same is true for different CDKs [77]. Furthermore, one must take into account that some cyclin-CDK complexes may play a tissue specific role instead of a universal role on the cell cycle.

$D_1^{-/-}$	Viable Impaired Mammary epithelial proliferation.	Fantl, 1995
$D_1^{-/-}$ $D_2^{-/-}$ $D_3^{-/-}$	Mice die in mid gestation due to cardiac abnormalities. MEFs proliferate normally, but show delayed response to mitogenic stimuli	Kozar, 2004
$E_1^{-/-}$	Viable	Genng, 2003
$E_1^{-/-}$ $E_2^{-/-}$	Embryonic lethal at E11.5 due to severe anemia; defect in endoreplication. MEFs proliferate slowly and fail to exit quiescence into cell cycle	Parisi, 2003
$A_2^{-/-}$	Embryonic lethal at implantation	Murphy, 1997
$B^{-/-}$	Embryonic lethal at E10.5	Brandeis, 1998
$Cdk_4^{-/-}$	Viable. Mice have reduced life span due to diabetes.	Rane, 1999
$Cdk_4^{-/-}$ $Cdk_6^{-/-}$	Embryonic lethal at E14.5. Mice die due to defects in hematopoiesis.	Malumbres, 2004
$Cdk_2^{-/-}$	Viable; reduced body size. Male germ cells die at pachytene and female ger cell die during diplotene stage of meiosis.	Berthet, 2003
$Cdk_2^{-/-}$ $Cdk_4^{-/-}$	Mice die during embryogenesis due to severe heart defects. MEFs show decreased cell proliferation and enter senescence prematurely	Berthet, 2006
$Cdk_2^{-/-}$ $Cdk_4^{-/-}$ $Cdk_6^{-/-}$	Embryos die at E15.5. Liver showed reduced cellularity.	Santamaria, 2007
$Cdk_1^{-/-}$	Not viable. Cdk1 is essential for the early stages of embryonic development.	Santamaria, 2007

Table 1. Summary of mouse knockout studies
Adapted from Malumbres and Barbacid, Nat Rev Can, 2008

Knockout studies of the genes encoding D-type cyclins have indicated that their individual functions are essential in specific tissue types. cyclin D1 knockout causes aberrant

mammary epithelial proliferation during pregnancy and cyclin D2 knockout impair beta cell proliferation [78, 79]. Knockout animals of the individual D type cyclins are viable. Animals with all three D-type cyclins knocked out are not viable but the embryos live until stage E16.5 and die due to defective erythropoiesis [80]. Individual CDK4 or CDK6 knockout animals also show tissue specific phenotypes similar to those observed in animals without D-type cyclins. The most drastic consequences due to loss of cyclin D-CDK4/6 complexes occurred in tissues with a high proliferative demand [81-83]. Additionally, as cyclin D-CDK4/6 complex is a major intracellular effector of extracellular mitogenic stimulus, cells that lack D-type cyclins or CDK4/6 function fail to respond as efficiently to mitogenic stimulation. What is interesting though is that the cells that do respond to mitogenic stimulus in the absence of D-type cyclins do so with the same kinetics as the control cells, indicating that D-type cyclins biggest function is the initial response to mitogenic stimuli [80, 83].

Similarly, knockout studies with E-type cyclins and CDK2 indicate that these proteins are not essential for G1-to-S progression in all cells, nor for murine embryogenesis or post-natal development. CDK2 null animals are viable, albeit sterile [84, 85]. Indeed, CDK2 knockout animals are the least

affected of all cyclin and CDK knockout animals. E-type cyclin knockout animals suffer decreased fertility. Embryos without both E-type cyclins died at stage E11.5 due to placental failure [86, 87].

Taken together, the knockout studies suggest that individual G1/S cyclins and CDKs are not essential for development and proliferation. Different CDKs appear to compensate for the loss of others during mouse development. CDK2/CDK4 double knockout mice die during embryogenesis and MEFs lacking both CDKs show decreased pRb phosphorylation, reduced expression of E2F-target genes, and decreased cell proliferation[88]. Thus, while knockout of either CDK2 or CDK4 does not greatly affect cell proliferation, knockout of both reduced viability and cellular proliferation, suggesting cooperativity between these CDKs. Ultimately, the knockout studies have identified a few essential elements to the cell cycle. Namely, knockout cyclin A2 causes early embryonic lethality (E5.5) and that loss of CDK1 is not viable (Murphy, 1997) [89]. Additionally, replacement of both copies of CDK1 gene with copies of CDK2 was not enough to rescue the cells – indicating that CDK2 cannot compensate for loss of CDK1 [90].

Cell Cycle and Cancer

The hallmarks of cancer: uncontrolled proliferation, genetic instability and aberrant chromosome segregation often reflect deregulation of genes involved in cell cycle control. Most of the key cell cycle regulators have their activity perturbed in many aspects of neoplasia. Despite their redundancy and lack of significant phenotypic changes in knockout studies, cancers are replete with aberrant expression and persistent cyclins. It is noteworthy in this regard, that while loss of a cyclin or CDK may be compensated during embryogenesis, somatic alterations causing and increase or interference with cyclin/CDK function in mature mammalian cells has important consequences on cell cycling.

Increasing evidence suggests that the cyclins, CDKs and CDK inhibitors are either themselves targets for genetic change in cancer or are disrupted by other oncogenic events [91]. Both Cdc25A and Cdc25B are overexpressed in cancers [92, 93]. D-cyclins are commonly found to be overexpressed in tumors, often associated to chromosomal alterations including translocations and gene amplifications [94-97]. Breast tumors show a particularly high incidence of cyclin D1 overexpression in approximately 50% of cases. Cyclins D2 and D3 have also been found amplified and overexpressed in

different types of human cancer[98, 99]. Mammary specific transgenic cyclin E and cyclin D1 overexpression in mice cause mammary hyperplasia and adenocarcinomas [100, 101]. Like their regulatory partners, CDK4 and CDK6 are also target of genetic alterations in cancer [102]. CDK4 is amplified and overexpressed in a variety of tumors from different cellular origins [100, 103, 104]. Additionally, CDK4/6 function can be increased by the loss or silencing of the inhibitor $p16^{INK4A}$ [105]. MEFs lacking all the three D-type cyclins, or CDK4, are resistant to transformation induced by oncogene transfection [80, 106].

There is abundant evidence for aberrant activation of CDK2 in human carcinogenesis or progression [91, 107]. CDK2 gene amplification and CDK2 overexpression and activation are observed in primary colorectal [108, 109], lung [110] and ovarian carcinoma [111]. Cyclin E deregulation is directly implicated in cancer [101]. Unusually high and persistent levels of cyclin E have been observed in human tumor cells, especially in the most aggressive cancers [112]. Cyclin E overexpression and CDK2 activation contribute to karyotypic abnormality and genomic instability [113, 114] though interference with the pre-replication complex assembly [115]. Deregulation in some naturally occurring cancers has been associated with mutations in the hCDC4 gene [113, 116] leading

to the lack of the ability of cells to degrade cyclin E shortly after S-phase entry.

The CDK inhibitors appear to function as tumor suppressors. Genetic inactivation or loss of p16 is frequent in human cancers [117]. Although p27 mutations are rare we and others reported that accelerated ubiquitin-mediated p27 degradation is frequent [118, 119] and is associated with a poor prognosis in breast, colon, lung and prostate carcinomas [120-125]. The reduced levels of p27, observed in up to 60% of human tumors [119], are correlated with cyclin E-CDK2 activation in the affected tumor tissue [120].

Regulation of the PI3K Pathway and Cancer

The G_1 to S phase transition of the cell cycle is of particular interest to cancer research since this step is usually misregulated in cancer cells [91, 92, 107, 126]. The PI3K pathway is activated during G_1 and is required for S phase entrance. Protein kinase B (PKB), also called AKT, is a key downstream effector of PI3K. The PI3K pathway is activated during G1 [127] and is required for S phase entrance [128-130]. Thus, PI3K activation is temporally linked to the restriction point that coincides with cyclin E-CDK2 activation. The PI3K pathway is frequently deregulated in human cancers through oncogenic activation of receptor

tyrosine kinases, autocrine production of their ligands, Ras mutation, PTEN inactivation and mutational activation of genes encoding PI3K components [131, 132]. (See Figure 3). The constitutive activation of the PI3K pathway, and the subsequent activation of its downstream effectors, is thus an important mediator of uncontrolled cell cycle progression during oncogenesis [133]. Recent work from the Slingerland lab has shown that phosphorylation of p27 by AKT, or other AGC type I kinases, causes that protein to switch roles: from a cell cycle inhibitor – where it binds and inhibits the activity of Cyclin E/CDK2 – to a mitogen and possibly a motility factor – where it is primarily localized to the cytoplasm, is acts as an assembly factor and possibly a nuclear targeting complex for Cyclin D/CDK4 complexes [134]. Moreover, PI3K effector-mediated phosphorylations of p27 promote its binding to RhoA leading to an increase in cell motility, invasion and metastasis [134].

During my thesis work, I have sought to determine if the PI3K pathway, and AKT in particular play a role in CDK2 regulation. Unpublished preliminary data from the Slingerland lab suggested that CDK2 contains an AKT substrate motif and that AKT formed a complex with CDK2. My research investigated further the consequences of PI3K

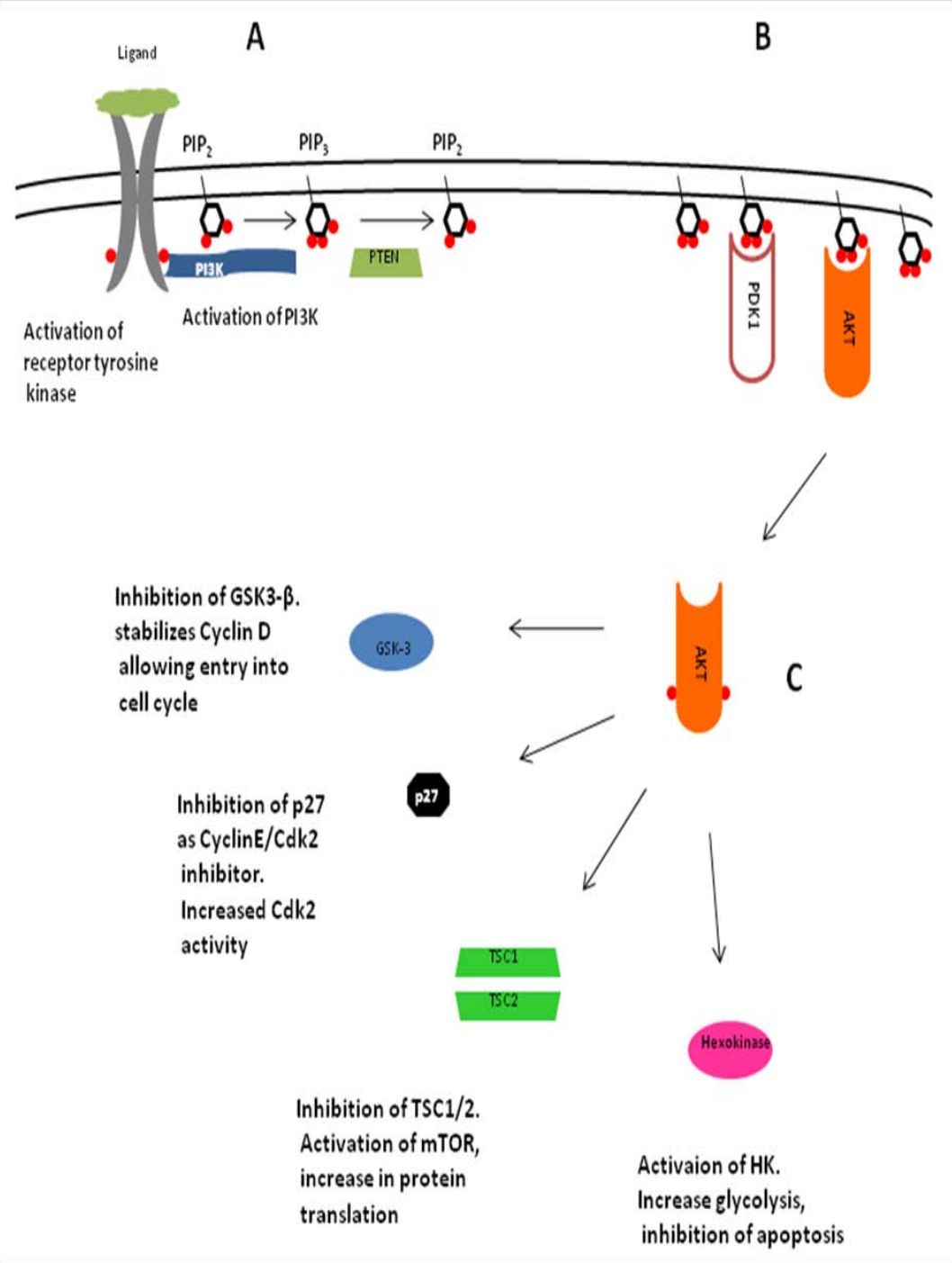

Figure 3. PI3K signaling pathway. Binding of ligand (**A**) causes the activation of receptor tyrosine kinase which in turn activate PI3K, causing an accumulation of PIP_3. This can be reversed by PTEN activity. PIP_3 serves as a docking site for PDK_1 and Akt (**B**). Akt is activated via phosphorylation through PDK_1 and PDK_2. Akt has many downstream effectors (**C**) that control cell cycle progression, protein translation, apoptosis and metabolism.

activation, and subsequently of AKT, on CDK2 phosphorylation, activation and its function to promote G1-to-S phase progression. The work described here outlines the investigation of a novel mechanism through which AKT, a major downstream effector of the PI3K pathway, regulates CDK2 activity. This is especially germane to human cancers since it provides a novel mechanism through which mitogen-mediated PI3K activation may regulate the accumulation of cyclin-CDK2 complexes and contribute to the regulation of cyclin E-CDK2 activity in normal cells and accelerate G1-S phase progression in human cancers.

Chapter 2

MATERIALS AND METHODS

Methods used for mammalian cell lines

Cell culture

MCF-7 and MEF cells were grown in Dulbeco's improved Eagle's medium (DMEM) supplemented with L-glutamine and 5% fetal bovine serum (FBS) as described [135]. Where indicated, cells were grown to 50% confluence and then treated with 10 μM LY294002 (Roche Scientific) or vehicle (DMSO) control prior to protein and cell cycle analysis.

Cell lysis

Cells were lysed in 0.5% NP-40 buffer (0.5% Nondet P-40, 50 mM Tris pH 7.5, 150 mM NaCl) supplemented with protease inhibitors (1 mM PMSF, and 0.02 mg each of aprotinin, leupeptin, and pepstatin per mL) and then centrifuged at high speed for 15 minutes. Supernatant was then collected and analyzed for protein concentration.

Immunoprecipitation and western blotting

Immunoprecipitation and immunoblotting experiments used 500μg and 50 μg of protein lysates respectively unless otherwise indicated. All proteins precipitated in IP-Western blot have been shown not to bind to non-specific antibody controls. Antibody alone controls were run

alongside all immunoprecipitates and equal protein loading for all Western blots was verified by amido black staining.

Antibodies

Cyclin E mAb was a gift from E. Harlow (Harvard Medical School, Boston, MA). Antibodies against phospho AktS473, total Akt, phospbo-Akt substrate and phospho-CDK2T160 and p27 were obtained from Cell Signaling Technology. Antibodies against p21, p27 (C-19), and CDK2 (M-2), Cyclin E (HE111 and C-19) and cyclin A (BF683 and M20) were obtained from Santa Cruz Biotechnology.

Cell cycle synchrony

MCF-7 cells were G_0 arrested by transfer to mediate containing 0.1% cFBS for 48 hours then released into cell cycle by the addition of full serum (10% FBS). Cells were recovered at intervals for protein and cell cycle assays.

Flow cytometry

Cells were pulsed with 10 μM bromodeoxyuridine (BrdU) for two hours and processed for flow cytometry as described [136].

Methods used for S. *cerevisiae* cell lines

Generation of Cdc28 mutant strains

The yeast strain with an HA-tagged CLN2 (CWY626, provided by Dr. K. Wittenberg, The Scripps Research Institute, CA) was transfected with pRS415 vector containing Cdc28wt, cdc28-S46A or cdc28-S46E (provided by Dr Marshak, give institute). These strains were subjected to PCR-mediated gene knockout [137] using the CloNAT dominant drug marker cassette (provided by Dr S. Lemmon, U of Miami, FL) and the appropriate primers (Table 2). Verification of the knockout was performed using PCR and the appropriate primers (Table 2).

Cdc28	MATa *CDC28::NAT*[WT *CDC28* in pRS415] *ade1 leu2-3, 112 his2 trp1-1 ura3Δns bar1Δ cln2::CLN2-3xHA::URA3* pRS415-*CDC-28*
Cdc28-S46A	MATa *CDC28::NAT*[*CDC28-ala* in pRS415] *ade1 leu2-3, 112 his2 trp1-1 ura3Δns bar1Δ cln2::CLN2-3xHA::URA3* pRS415-*cdc28-S46A*
Cdc28-S46E	MATa *CDC28::NAT*[*CDC28-glu* in pRS415] *ade1 leu2-3, 112 his2 trp1-1 ura3Δns bar1Δ cln2::CLN2-3xHA::URA3* pRS415-*cdc28-S46E*

Table 2. The CWY626 yeast strain was used as the parental strain for derivation of the Cdc28 genotypes used in the present study

Cell lysis

Cell lysates were prepared by vortexing in lysis buffer (0.1 % NP40, 250 mM NaCl, 50 mM NaF, 5 mM EDTA and 50 mM Tris-HCl pH 7.5) in the presence of glass beads. Crude clarified lysates were recovered after centrifugation at 15

000 x g for 15 min. Lysate protein concentrations were determined by Bradford assay (Bio-Rad); lysates typically contained 10-20 mg/ml of total protein.

Immunoprecipitation and western blotting

Direct immunoblot analysis used 100 ug of total protein/lane. Cell lysates were diluted into protein sample buffer on ice and boiled immediately before loading on 12% polyacrylamide-SDS gels. Immunoprecipitations were carried out using 1mg of protein and 500ng anti-Cdc28 antibody (Sigma). Immune complexes were collected on protein A - Sepharose beads at 4'C for 1 h. For detection of immunoprecipitated proteins, beads were centrifuged (1000 g for 5 s), washed four times with lysis buffer and boiled in protein sample buffer immediately before SDS-PAGE. CLN2 immunoblots used were 3F10 (Roche) and Cdc28 was detected with anti-PSTAIRE mAb provided by M. Yamashida (Okazaki, Japan). Primary and secondary antibodies were diluted 1:200 and 1: 10 000, respectively.

Cell Cycle Synchrony

Synchronization by release from an □-factor block was done by arresting cells (typically 400 ml grown in YPD to 8 x 10^6 cells/ml) for 3h at room temperature with 5 μM α-factor,

washing twice with 100 ml pre-warmed YPD and resuspending in the original volume of YPD. At 5 min intervals aliquots were removed, rapidly pelleted and washed once with ice-cold water before lysis flash freezing in liquid nitrogen. Typically 30 ml of culture were used for protein lysates and 1 ml was used to confirm arrest by microscopic examination and FACS analysis.

Flow Cytometry

10^7 cells from an exponentially growing culture were pelleted and fixed with ice-cold 70% EtOH. Fixed cells were then washed twice with 4mL of wash buffer (50 mM Na citrate, pH7.4). Cells were subsequently incubated in wash buffer with 0.1 mg/ml RNase A for 2 h at 37 °C. Cells were then washed 2 times with 5 mL wash buffer and treated with 0.5 ml wash buffer containing 2 μM Sytox Green.

Cdc28 kinase assays

Active Cdc28 was immunoprecipitated from cleared yeast cell lysate. For quantitative kinase reactions, at least 300 □g of cleared cell lysate was used to precipitate active Cdc28 with an anti-Cdc28 antibody (Sigma). Immunoprecipitates were washed twice with kinase reaction buffer (25 mM Tris, pH 7.5, 5 mM Glycerol 2-phosphate, 01 mM

Sodium Vanadate, 10 mM MgCli, 1 mM DTT). Reactions were carried out at 30°C for30 minutes in the presence of 10□Ci [y-^{32}P] ATP and 5□g histone Hl; products were resolved by SDS-PAGE and incorporated radioactivity quantified by phosphoimager analysis of dried gels as described previously by [138].

Recombinant protein expression and assembly assays

Cloning and Site directed mutagenesis of CDK2

The full length human CDK2 cDNA sequence was excised pCMV-CDK2. The cDNA sequence was verified by sequencing and then sub-cloned into the pET41b vector using the restriction endonucleases BamHI (5’) and XhoI (3’). Site directed mutagenesis of the verified pET25b-CDK2 vector was performed according to manufacturer’s instruction using the primers designed to generate mutant CDK2 alleles bearing T39A or T39E.

Bacterial protein expression and purification

Recombinant His-tagged CDK2-WT, CDK2-T39A, CDK2-T39E, were cloned into pET15b vector and transformed into *E.coli* BL21 (DE3) cells, grown in LB media to mid exponential phase and induced with 1 mM IPTG 6 hours at at 32°C. Cells were pelleted and resuspended in lysis buffer (50mM tris-HCl,

pH8.0, 250mM NaCl, 1% triton x-100). Cells were lysed using a French press and lysate was cleared via centrifugation. The soluble fraction was then applied to a 1 mL TALON column (Clontech). The Loaded column was washed 3 times with 5 bed volumes of lysis buffer. Loaded beads were then washed with 5 bed volumes of Buffer I (50mM tris-HCl, pH8.0, 250mM NaCl, 10% Glucose, 1% triton x-100, 50mM imidazole). Protein was eluted from the column by buffer II (50mM tris-HCl, pH8.0, 250mM NaCl, 10% Glucose, 1% triton x-100, 100mM EDTA). Eluate was dyalized against 50 volumes of dialysis buffer, changing dialysis buffer twice (50mM tris-HCl, pH8.0, 200mM NaCl, 10% Glucose, 1% triton x-100). Dialysed solution was concentrated to 0.5 mg/mL.

pBABE retroviral production and infection

CDK2-WT, CDK2-T39A and CDK2-T39E were sub-cloned from the pET15b plasmids used for recombinant protein expression using the restriction enzymes SnaBI and EcoRI. DNA fragments were then gel purified and ligated into pBABE-puro vector overnight at 16°C. Ligation reactions were then transformed into DH5□ cells and insertion was verified by gel electrophoresis and by DNA sequencing. pBABE-CDK2-WT, pBABE-CDK2-T39A and pBABE-CDK2-T39E vectors were transfected into Phoenix-AMPHO cells using Lipfectamine

Plus Reagent. About 48-72 hours post-transfection, supernatant containing the virus was centrifuged at low speed and then filtered through a 0.2 µm filter. MEF$^{cdk2-/-}$ cells were then transduced with the cleared, virulent PHOENIX cell media. 72 hours post-infection cells were selected with puromycin for two days. Cells were subsequently cultured in normal media and collected for protein analysis.

Baculoviral protein production and purification

Baculoviral supernatant for recombinant Flag-cyclin A and His-Cyclin E were kindly provided by L, Hengst, Give Institute, Austria. Flag-Cyclin A and His-Cyclin E were harvested from infected Sf-9 cells and the protein was purified using a TALON column (Clontech) as described above for recombinant His-CDK2.

AKT kinase assays

Recombinant active Akt was obtained from Millipore/Upstate. Reaction conditions (time and Akt concentrations of 20-200 ng) were varied to determine the linear range of kinase assay conditions. For the quantitative kinase reactions, 100 ng PKB (Millipore/Upstate) were incubated with 2.5□g recombinant His-CDK2 proteins in □20 kinase reaction

buffer (20 mM HEPES pH 7.4, 10 mM MgCl2, 10mM MnCl2, 1 mM dithiothreitol, 100 μM ATP). Reactions were carried out at 30°C for 20 minutes in the presence of 10 μCi [γ-^{32}P] ATP, products were resolved by SDS-PAGE and incorporated radioactivity quantified by phosphoimager. Akt was also immunoprecipitated with Akt-pS473 antibody (Cell Signaling Technology) from increasing amounts of asynchronous WM239 cell lysate and reacted with His-CDK2 proteins as above. Amounts of immunoprecipitated Akt, substrate and reaction duration were titrated to ensure that reactions were carried out in the linear range. Immunoprecipitated Akt was reacted with 2.5μg recombinant CDK2 proteins as above, and as in [135].

Cyclin-CDK2 assembly assays

To assay CDK2 binding to cyclins A and E, purified Cyclins E and A were first allowed to react with their respective antibodies (HE111 and BF468, Santa Cruz) in 400□l binding buffer (20mM tris-HCl, pH7.4, 10% Glucose, 1% triton x-100) for 40 minutes. 30 μL Protein A sepharose beads were then added to the reaction and incubated for 30 min. Beads were then washed twice with 400 L binding buffer to remove unbound cyclin/antibody. Beads were resuspended in a final volume of 300□L prior to the addition of recombinant

purified CDK2. These reactions were carried out in a 2:1 molar excess of cyclin over CDK2.

To assay the effect of CDK2 phosphorylation by Akt on cyclin-CDK2 assembly,recombinant CDK2 was used as substrate in an Akt kinase assay using conditions described above for one hour. The kinase reaction was boiled for 1 minute to inactivate Akt prior to the assembly reaction.

Two dimensional tryptic phosphopeptide mapping

Asynchronously proliferating cells or cells treated with 10 □M LY294002 for 48 hours were incubated in phosphate free medium containing 5% dialyzed FBS for 4 hours prior to metabolic labeling with 1 mCi of [γ^{32}P] orthophospbate (Amersham Pharmacia Biotech) per p100 dish for 3 hours at 37°C. Cells were lysed on ice-cold lysis buffer. Cell lysates were then pre-cleared with normal rabbit IgG prior to immunoprecipitation of CDK2 or HA-CDK2. The immunoprecipitates were resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a polyvinyl difluoride membrane and autoradiographed. CDK2 or HA-CDK2 bands excised from the membrane were blocked with 0.5% polyvinyl difluoride in 100 mM Tris (pH 7.6) at 37°C for 30 minutes and digested overnight with 10μM modified trypsin (Roche Diagnostics). The samples were then lyophilized and

treated with ice-cold performic acid [45□L of formic acid acid 5ml of H_2O_2 (37%)]. Lyophilized samples were re-suspended in 5ml of acidic buffer (aqueous solution containing 2.5% formic acid; 7.8% acetic acid; pH 1.9) and loaded onto cellulose thin layer plates. Electrophoresis was performed with pH 1.9 buffer for 1000V/hr using Hunter thin-layer electrophoresis system (HTLE-7000; CBS Scientific, Del Mar, CA). Ascending chromatography was performed using a phosphochromatography buffer (aqueous solution containing 37.5% n-butanol, 25% pyridine, and 7.5% acetic acid) for 24 hours. The plates were then air-dried and subjected to autoradiography.

Chapter 3

RESULTS

Figures 4 and 6 represent the work of Dr Jiyong Liang in the Slingerland lab. The experimental work of Figures 5, and 7-14 was planned and carried out by the PhD candidate, Thiago daSilva. Dr K. Drews-Elgar assisted in the cell synchronization and recovery of lysates for Figure 7. Dr M. Tan assisted with work of Figure 8.

CDK2 forms complex with Akt

Akt is a key downstream effector of the PI3K pathway. Akt phosphorylates substrates at a consensus sequence K/RXRXXS/T [139]. Our *in silico* search revealed that CDK2 has a putative Akt consensus motif –KIRLDT-, spanning residues 33-39, that is highly conserved in all known CDK2 species, in human CDK1 (S39) and in CDK6 (T49) (Figure 4A). In the

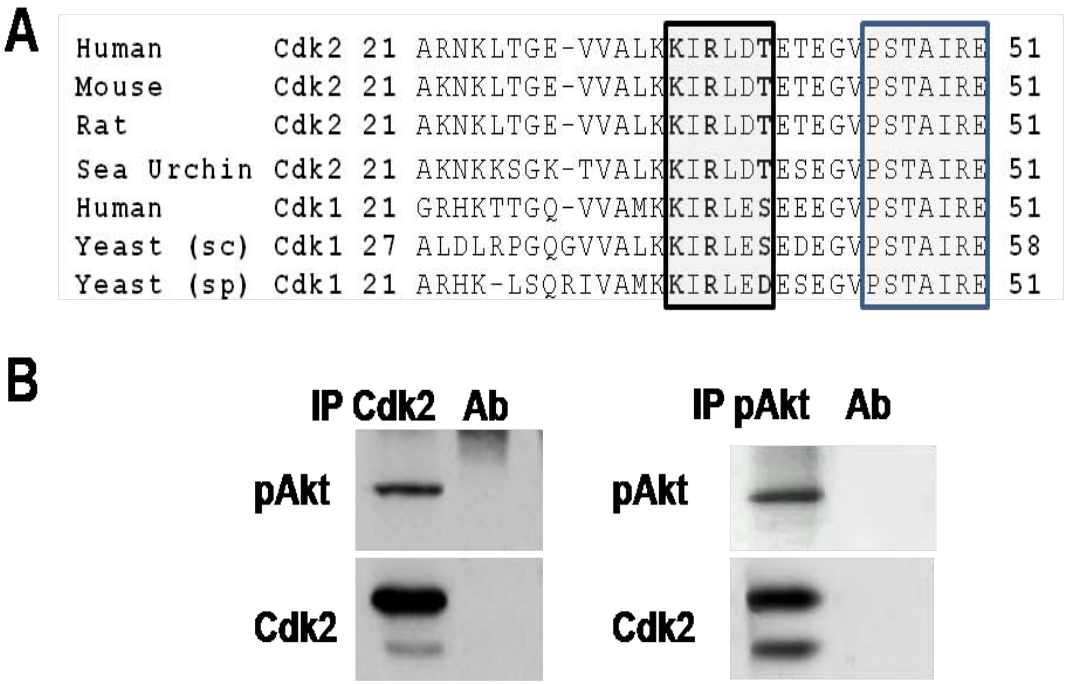

Figure 4. Cdk2 associates with Akt. **(A)** The Akt consensus sequence - K/RXRXXS/T- (enclosed in blacb box) is highly conserved from the budding yeast, *S. cerevisiae* (sc), to humans, this site is present just preceding the PSTAIRE helix (enclosed in blue box). In the fission yeast *S. pombe* (sp), the S/T is replaced by D. **(B)** Cellular Cdk2 binds Akt. Cdk2 or active pAkt was precipitated from MCF-7 cells followed by blotting for Cdk2 and pAkt with a phospho Ser-473 specific Akt antibody. Ab indicates antibody only control A sepharose beads without added lysate.

budding yeast CDK homologue, Cdc28, this site is on the serine 46. In *S pombe*, the homologous site in Cdc2 bears an aspartate carrying a net negative charge. This motif is located in the N-terminus and it precedes the PSTAIRE helix sequence, which is a crucial docking site for cyclins.

As for many kinase-substrate pairs, Akt is known to bind to certain cellular substrates [140]. Having identified a putative Akt-phosphorylation site in CDK2, we next tested if Akt formed a complex with CDK2. This was assayed by immunoprecipitation with antibodies against active Akt (pAkt denotes S473-phosphorylated Akt detected with phospho-specific antibody) or CDK2 followed by Western blotting for the associated protein using lysates from asynchronously proliferating MCF-7 cells. CDK2 precipitates contained pAkt. Similarly, CDK2 was detected in pAkt immunoprecipitates (Figure 4B).

Of particular note was the difference in the amount of T-loop phosphorylated CDK2 (the faster migrating band of CDK2 [57] present in total CDK2 precipitates versus that in complex with pAkt. CDK2 precipitates contained only a small amount of the faster mobility – active – CDK2, while pAkt-bound CDK2 showed a greater proportion of CDK2 in the faster migrating form. Asynchronous cells contain most of the CDK2 pool in the monomeric form, that is, not in complex

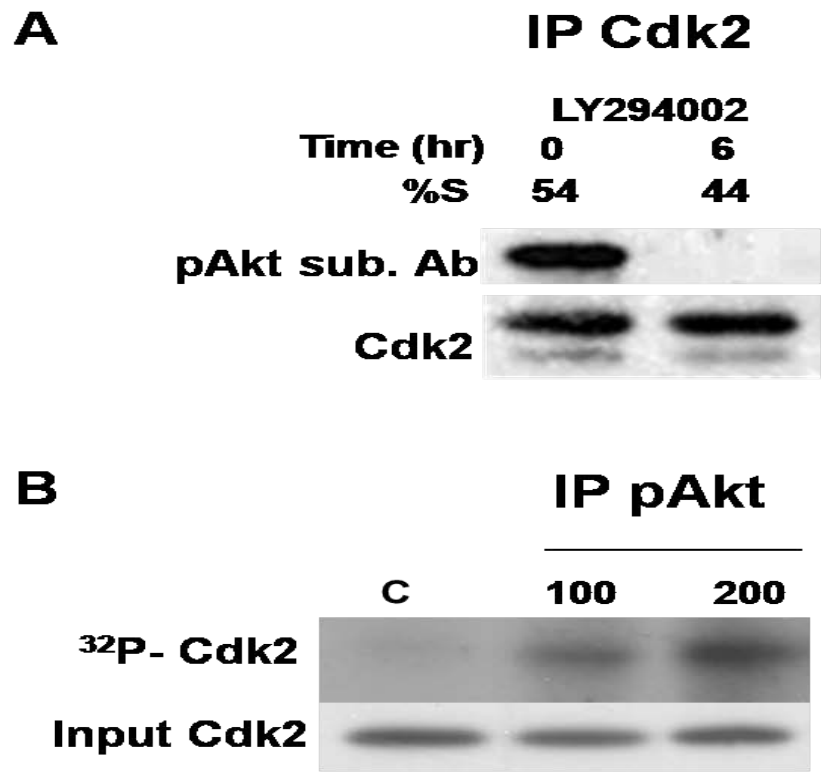

Figure 5. Cdk2 is phosphorylated by Akt in vivo and in vitro. (A) MCF-7 cells were treated with or without LY294002 for 6 hours and Cdk2 was precipitated; complexes were resolved and blotted with antibody specific for a phosphorylated Akt consensus motif (pAkt substrate) and then stripped and re-probed for Cdk2. **(B)** Akt phosphorylates Cdk2 in vitro. His-Cdk2 was reacted with γ-^{32}P-ATP and immunoprecipitated cellular active Akt (pAkt) or normal IgG control (C) for 30 minutes under Akt kinase reaction conditions. Autoradiograph showing incorporated radioisotope in Cdk2 substrate is on the top panel; Input Cdk2 is shown in the bottom panel.

with cyclin and largely unphosphorylated at T-160. Our observation that pAkt precipitates a greater portion of CDK2-pT160 suggested that Akt may be involved in CDK2 activity or regulation.

CDK2 is phosphorylated by Akt *in vivo* and *in vitro*

Previous work has shown that Akt, and other AGC type I kinases, regulate cell cycle progression by phosphorylating p27 and other cell cycle regulators [55]. We next assayed if CDK2 is a substrate of Akt. CDK2 precipitates from proliferating cells were resolved and immunoblots were reacted with an antibody that detects proteins containing a phosphorylated Akt consensus motif (phospho-Akt substrate antibody). A 34 kD band was detected by CDK2 immunoprecipitation followed by western blotting using this phospho-Akt substrate antibody (Figure 5A, top blot). Treatment with 10 μM LY29004, a PI3K inhibitor, leads to a loss of phosho-Akt substrate antibody reactivity with CDK2 immunoprecipitates. When these blots were stripped and re-probed with CDK2 antibody, the 34 kD band identified by the phospho-Akt substrate antibody co-migrated precisely with CDK2 (Figure 5A, bottom blot). To verify that the 34 kD protein that reacted with the phospho-Akt substrate antibody was indeed CDK2, CDK2 immunoprecipitates were boiled in 1% SDS to dissociate CDK2-bound proteins. Subsequent precipitation of monomeric CDK2 retained reactivity with the phospho-Akt substrate antibody (data not shown).

Additionally, we used recombinant His-tagged CDK2 in an *in vitro* kinase assay with active Akt and demonstrated that

CDK2 is indeed an Akt substrate. When increasing amounts of kinase were used, radioactivity uptake in substrate increased (Figure 5B, top). Equal substrate input is shown (Figure 5B, bottom). Reaction conditions were titrated to ensure linear kinase activity for conditions of time, temperature and substrate used (data not shown). Reaction of recombinant CDK2T39A with AKT in vitro yielded no phosphorylation at T39 (data not shown).

2-D mapping of CDK2 pT39 site

Two-dimensional tryptic mapping of ^{32}P-orthophosphate labeled cellular CDK2 provided further evidence that CDK2-T39 is phosphorylated *in vivo*. Three major phosphopeptides were detected in CDK2 from asynchronously proliferating cells (Figure 6A).

The most strongly phosphorylated peptide contained only phosphotyrosine by phosphoamino acid analysis (data not shown). Its migration is consistent with the pY15-containing peptide as shown before [57]. Our 2D map also identified a phosphopeptide migrating in the position shown previously to contain pT160 [57]. Its phosphorylation was reduced by PI3K/AKT inhibition with 10 μM LY294002. A previously unidentified CDK2 peptide was strongly

phosphorylated in WM239 cells (see arrow, Figure 6 A); LY294002 treatment for 48 hours diminished phosphorylation of this novel phosphorylation site.

To test the effect of T39 mutation on the novel peptide, a C-terminal HA-tagged CDK2T39A (HA-CDK2T39A) vector was generated by site directed mutagenesis, converting T39 to alanine. CDK2T39A and wild type CDK2 (HA-CDK2WT) were stably transfected into MCF-7 cells. Stable cell lines bearing either CDK2-Wt or CDK2T39A were phosphate labeled. Anti-HA immunoprecipitation followed by two-dimensional tryptic mapping demonstrated that the novel peptide was not phosphorylated in labeled HA-CDK2T39A (Figure 6B). Not only was the novel phospho-peptide lost in the HA-CDK2T39A bearing cells, the site corresponding to the T160 phospho-peptide was also strongly attenuated, suggesting that T160 phosphorylation may be dependent on T39 phosphorylation. These data are consistent with a PI3K/Akt dependent CDK2 phosphorylation at T39. Additionally, the tandem loss of CDK2 T39 and T160 phosphorylation suggests that the CDK2 T39 phosphorylation may influence the regulation of CDK2 activity.

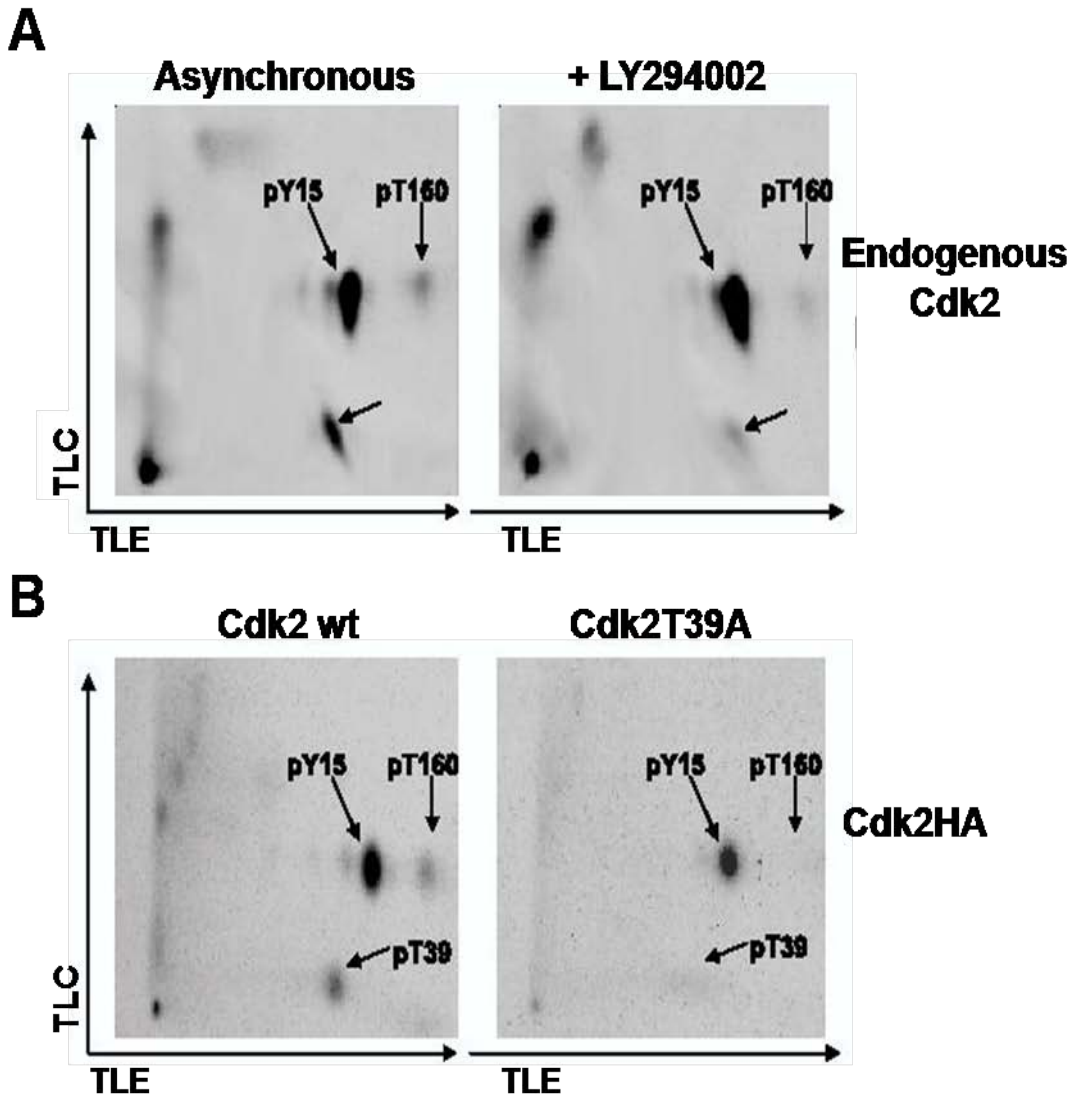

Fig 6. A T39-dependent phosphopeptide in Cdk2 is reduced by PI3K inhibition. Cells were ^{32}P-orthophosphate labeled for 3 hours, followed by Cdk2 immunoprecipition. Precipitates were resolved by SDS PAGE and two-dimensional tryptic maps carried out by thin layer chromatography (TLC); and thin layer electrophoresis (TLE); arrows indicate the origins of 2D migration. **(A)** Phosphopeptide mapping of endogenous Cdk2 from WM239 cells treated with (+) or without (-) LY294002 (20μM) prior to and during ^{32}P labeling. **(B)** Phosphopeptide mapping of HA-Cdk2 from WM239 cells transfected with HA-Cdk2WT or HA-Cdk2T39A, respectively, 24 hours prior to ^{32}P-orthophosphate labeling. Phosphopeptides are indicated by arrows.

Timing of CDK2T39 phosphorylation

Our next goal was to determine the timing of CDK2T39 phosphorylation during cell cycle progression. The PI3K signaling pathway triggers a network of events that positively regulate G1/S cell cycle progression [141]. The PI3K pathway and its downstream effector, Akt, are stimulated within hours when serum-starved, quiescent cells are induced to enter cell cycle by re-addition of mitogens [55]. The relative timing of CDK2T39 and CDK2T160 phosphorylations and cyclin-CDK2 complex formation and activation was assayed during G0-to-S phase progression. We reasoned that by observing changes in CDK2T39 phosphorylation we could gain deeper insight into the role this of phosphoryation vis-a-vis cell cycle progression, cyclin binding and CDK2 activation.

MCF-7 cells were deprived of serum for 48 hours and then stimulated to exit quiescence by serum re-addition. Akt activation peaked between 4 – 6 hours after serum addition (Figure 7). Cyclin E levels were periodic and showed a peak cyclin expression at around eight hours. The appearance of CDK2pT160 was gradual, occurred after that of pAkt and showed a continuous increase intensity during G0-S. This gradual increase in CDK2pT160 phosphorylation is in agreement with our current understanding of CAK activity

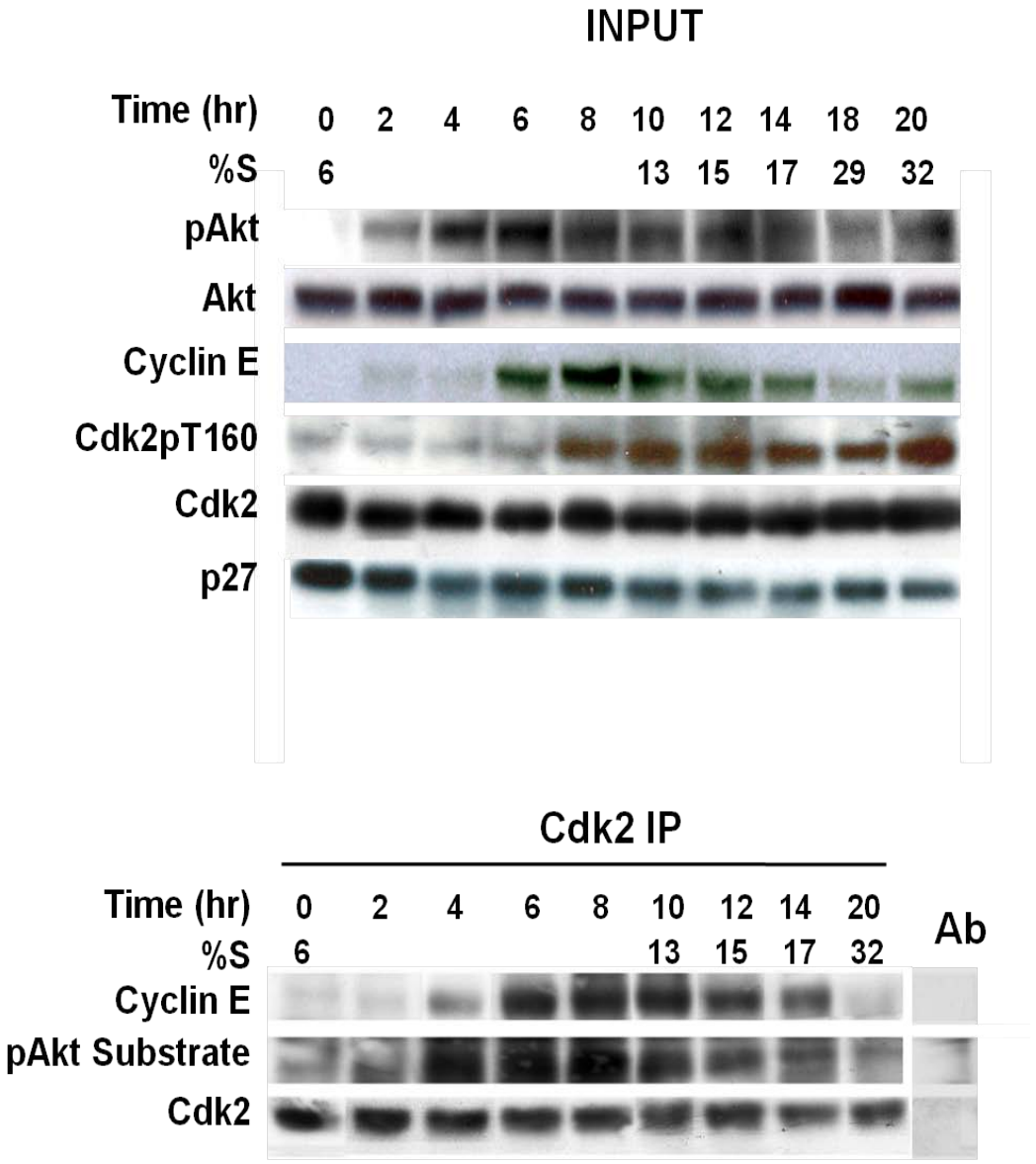

Figure 7. Akt activation precedes cyclin E-Cdk2 assembly in G_1. Estrogen and growth factor-starved G_0 arrested MCF-7 cells were stimulated to enter cell cycle at time zero. At intervals thereafter, cells were collected for BrdU labeling, flow cytometry, and protein analysis. (**A**) Protein levels were detected by Western blotting. (**B**) Cdk2 immunoprecipitation shows that Cdk2T39 phosphorylation precedes cyclin binding and the increase in Cdk2T160 phosphorylation. "Ab only" indicates reaction of precipitating antibody with protein A-sepharose without lysate to control for reactivity of immunoblotting antibody with heavy or light chain of the antibody used in immunoprecipitation (Ab). The timing of Cyclin E-Cdk2 kinase activity across this time course will be added to this figure prior to the thesis defense.

and function [64]. The timing of CDK2 phosphorylation was assayed by probing CDK2 immunoprecipitates with the phospho-Akt substrate antibody. We observed a periodic 34Kd band whose intensity that closely matched the timing of Akt activation. CDK2T39 phosphorylation preceded peak cyclin expression and cyclinE-CDK2 binding. The temporal relationship of Akt activation, CDK2pT39 appearance and CyclinE-CDK2 catalytic activation will be shown in the final version of the thesis. Thus CDK2 phosphorylation preceded both CDK2 phosphorylation at T160 and cyclinE-CDK2 binding during G0-S-phase progression.

The loss of CDK2pT39 also preceded the loss of CDK2pT160 and the loss of cyclin E-binding to CDK2 when asynchronous cells were treated with the PI3K inhibitor, LY294002. Loss of CDK2 reactivity with the phospho-Akt substrate antibody was notable by six hours (Figure 8). While there was little loss of cyclin E protein over 24 hours of LY294002 treatment, loss of CDK2-bound cyclin E was evident by 12 hours, concomitant with the loss of CDK2pT160 signal.

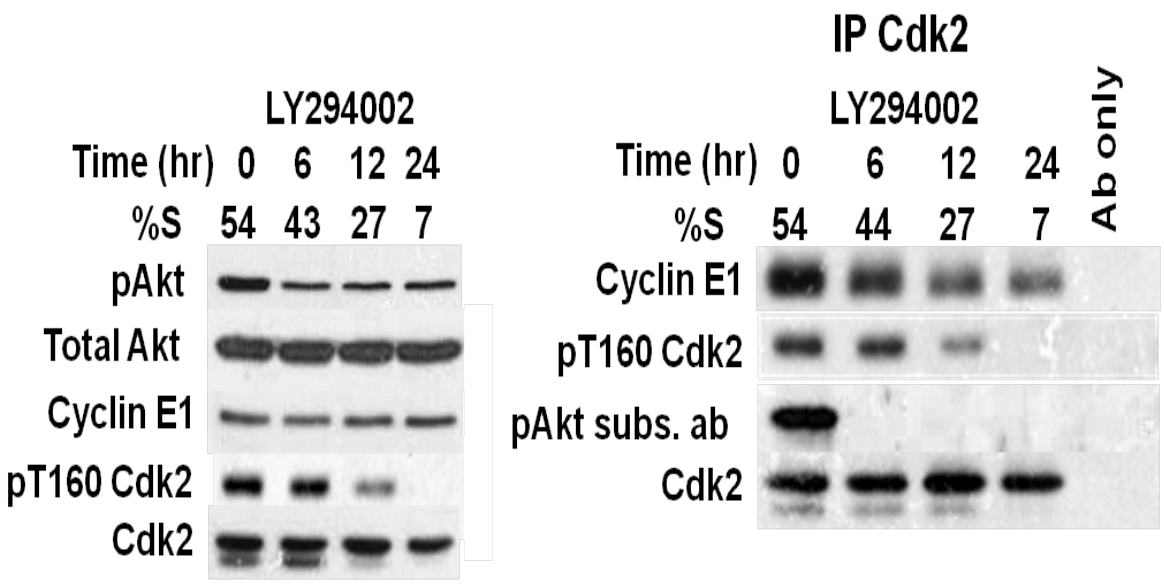

Figure 8. LY294002 treatment causes Cdk2 pT39 loss, cyclin dissociation. (**A**) Immunoblot of LY294002 treated cells. (**B**) Effects of PI3K inhibitor on Cdk2 complexes. After addition of LY294002, loss of Cdk2 reactivity with pAkt substrate antibody occurs within 6 hours and precedes the loss of pT160Cdk2. While total cyclin E levels are unaffected, LY causes loss of Cdk2-bound cyclin E and subsequent G_1 arrest.

When cells were recovered within minutes of LY294002 treatment, pAKt was reduced by 30 min and not detected at 1 hour, and loss of reactivity of CDK2 with anti-phospho-AKt antibody was notable within one hour (data not shown). The loss of CDK2pT160 was substantially delayed and notable only by 12 hrs of drug treatment. Altogether the rapid loss of CDK2T39 phosphorylation upon PI3K inhibition and the early appearance of the same phosphorylation event following AKT activation upon serum stimulation led us to

posit that this event precede CDK2 phosphorylation at T160 and may predicate cyclin-CDK2 complex formation or stability.

Mutation of CDK2T39 site alters cyclin binding *in vivo*

CDK2T39 is surrounded by charged residues that appear to stabilize two of the three β-strands adjacent to the PSTAIRE helix (residues 46-57) of CDK2 [142]. The region preceding the PSTAIRE helix has been shown to form a helical structure in when bound to cyclin E. Residues in the PSTAIRE region are on the interface of CDK2 that interacts with either cyclin E or cyclin A [73, 74, 142]. We postulated that T39 phosphorylation might affect the positioning of the PSTAIRE structure of CDK2 and influence CDK2 binding to its cyclin partners. Additionally, it has been recently shown that CDK2T160 phosphorylation is stabilized by cyclin binding. Therefore we postulated that the tandem loss of CDK2 T39 and T160 phosphorylations observed in the 2-D phosphopeptide mapping after PI3K inhibition could be due to changes in the ability of CDK2 to bind cyclin. Additionally the timing of CDK2T39 phosphorylation and dephosphorylation suggested that it may

play a role to regulate cyclin-CDK2 complex formation or activity.

To test if phosphorylation of CDK2T39 might affect steady state cyclin binding, $MEF^{CDK2-/-}$ cells were transduced with retroviruses that containing either pBABECDK2Wt, pBABECDK2T39A or pBABECDK2T39E. Upon selection for transfected cells and expansion of the culture, the CDK2-cyclin complexes were precipitated using anti-CDK2 antibodies and resolved on a SDS-PAGE gel. The relative amounts of cyclin that co-immunoprecipitated with each CDK2 isoform were detected by blotting (Figure 9A). Although similar amounts of CDK2 were precipitated from these cell lysates, the amount of cyclin A bound to CDK2 differed (Figure 9B). CDK2T39E precipitated more cyclin A than CDK2wt; and CDK2wt precipitated more cyclin A than CDK2T39A. It is noteworthy that the steady state level of cyclin A was also modestly increased in asynchronous CDK2T39E-bearing MEFs.

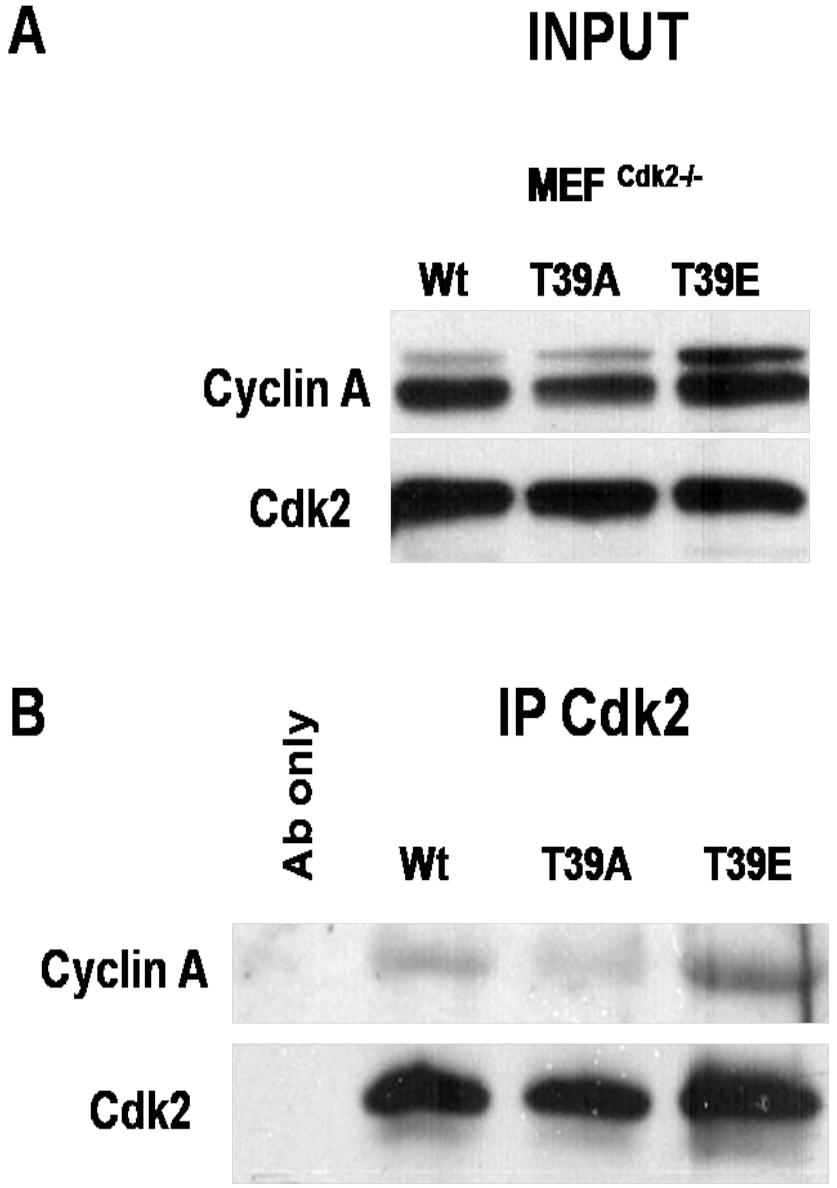

Fig 9. Cdk2T39A shows reduced coprecipitation of cyclin A *in vivo*. MEF$^{Cdk2-/-}$ cells were transfected with pBabe His-Cdk2WT, His-Cdk2T39A And His-Cdk2T39E. (**A**) Cyclin A and transfectedCdk2 were detected by immunoblotting. (**B**) Cyclin association with Cdk2 was detected by immunoprecipitation followed by immunoblotting for Cdk2 and Cyclin A

Mutations affecting the CDK2T39 site alter cyclin binding *in vitro*

The effects of CDK2T39 phosphorylation on cyclin binding were further evaluated by in-vitro binding assays. Recombinant cyclin A or cyclin E proteins were incubated with their respective antibodies (see Materials and Methods) and complexes pre-bound to Protein A sepharose beads and washed extensively to remove unbound cyclin. Equal inputs of the different recombinant CDK2 (WT, T39A and T39E) were then added to the pre-loaded protein A sepharose beads-antibody-cyclin complex for the times shown and then cyclin-bound CDK2 was assayed by immunoblotting after the complexes were resolved by SDS-PAGE and transferred (IP-blots shown for cyclin A-CDK2 in Figure 10A). For both cyclinA and Cyclin E- bound CDK complexes, CDK2T39E reached a maximal level of binding sooner than CDK2wt (Figure 10 B). CDK2T39E reached maximal cyclin A binding in less than 150 seconds whereas steady state CDK2wt binding was reached by 10 minutes (graphed as % input binding in Figure 10B-representative of three experiments). Similarly, the time required for maximal CDK2T39E binding to cyclin E was also shorter than CDK2wt (Figure 10C). All CDK2 isoforms reached a similar maximal level of binding, indicating that this phosphorylation may

not be required for binding. What is clear from our in vitro data is that this phosphorylation speeds up the rate of the reaction.

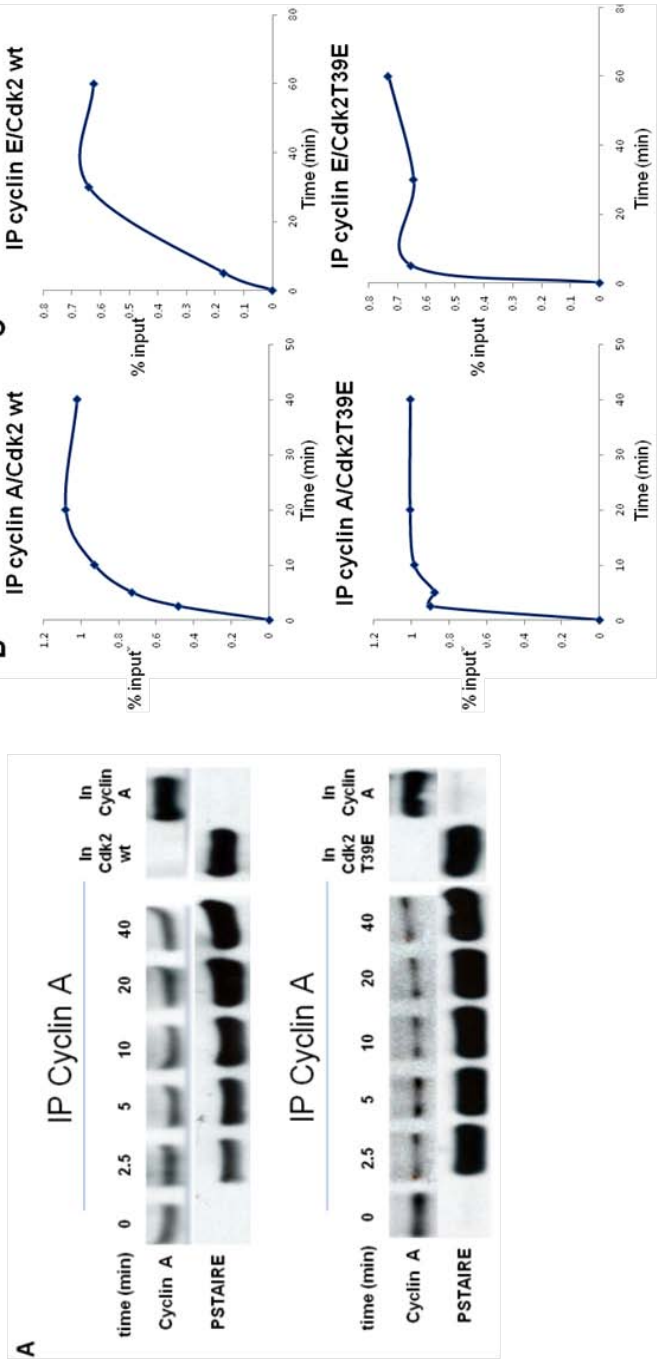

Fig 10. Phosphorylation of Cdk2 by Akt facilitates cyclin-Cdk2 assembly *in vitro* 10nM of purified His-tagged human Cdk2 and Cdk2T39E were reacted with 20 nM of purified recombinant human cyclin A or E. Levels of Cdk2-cyclin complexes formed were detected by cyclin immunoprecipitation followed by immunoblotting for Cdk2 using anti-PSTAIRE antibody. **(A)** Cyclin A- bound proteins shown in immunoblots with input into the binding reaction shown on the right. **(B)** Densitometric analysis of Cdk2 bound to cyclin A from IP-blots shown on left in (A) is graphed over time as a % of Cdk2 input into the reaction. **(C)** Densitometric analysis of Cdk2 bound to cyclin E is graphed as a % of the Cdk2 input into the reaction as in (B).

CDK2 phosphorylation by Akt increases the cyclin-CDK2 assembly rate in vitro

To further assay how phosphorylation of CDK2pT39 affects the rate of cyclin-CDK2 association, recombinant CDK2 was reacted with active Akt for 60 minutes prior to incubation with recombinant cyclin A for intervals between 1 and 30 minutes. Cyclin A-bound CDK2 was assayed by cyclin A immunoprecipitation followed by immunoblotting for CDK2. Akt treatment increased the rate of cyclin A-CDK2 complex formation. Two different controls were used: recombinant CDK2 was mock treated without addition of Akt (untreated), and in addition, a second CDK2 samples was treated with catalytically inactive Akt (produced by boiling active Akt for 30 min). CDK2 binding to cyclin occurred at similar rates in both control experiments, while pre-treatment of CDK2 with active Akt shortened the time required for maximal cyclin A-CDK2 association (Figure 11). It is noteworthy that all binding assays reached a maximum by 30 minute, with comparable steady state complex detected at these concentrations of input cyclin and CDK2. Data from Figures 10 and 11 together support the conclusion that CDK2 phosphorylation by Akt augments the rate of cyclin binding or impairs its dissociation, once bound.

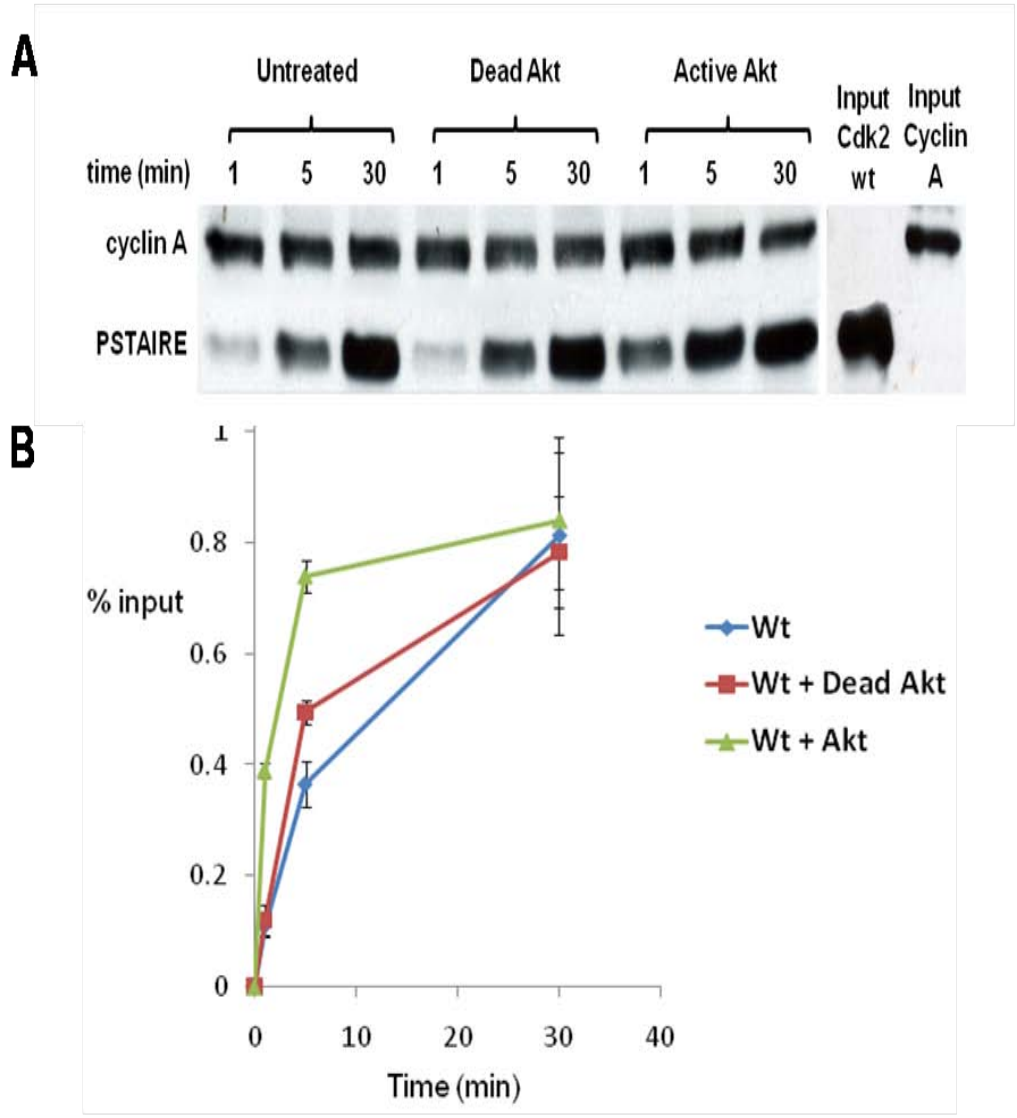

Fig 11. Phosphorylation of Cdk2 by Akt facilitates cyclin-Cdk2 assembly *in vitro*
Purified His-tagged human Cdk2 was reacted with either a Catalytically active or inactive Akt under kinase conditions. Pretreated Cdk2 (50 nM) was then incubated with 100 nM of purified recombinant human cyclin A for the indicated times at room temp. Cdk2-cyclin binding was assayed by cyclin immunoprecipitation followed by immunoblotting for Cdk2. (**A**) Immunoblot of binding assay shows that phosphorylation of Cdk2 by Akt increases the rate of cyclin binding. (**B**) Densitometric analysis of binding assay.

Replacement of Cdc28WT with cdc28-S46E shortens G_0-S transit time in *Saccharomyces cerevisiae.*

Our next goal was to determine if mutations affecting CDK2T39 would have an effect on cell cycle progression. My first attempts to test this using the stable CDK2 transfectants of the $MEF^{CDK2-/-}$ line were not successful because the MEFs did not show a reproducible transit time from quiescence to S phase when released from serum starvation. Moreover, the lack of reproducible differences between these CDK2 reconstituted MEF was likely due to the compensation by CDK1 for the lack of *CDK2* during embryogenesis of the *CDK2* null mice [84, 85]. Further attempts to knockdown CDK2 with adenovirus-cre in CDK2 floxed MEFs lines and retrovirally infect with CDK2WT or the T39 mutant alleles was again unsuccessful due to the lack of reproducible G0-S transit time in any one line. Therefore, we chose to use a simpler model system which contained only one G_1 CDK, the budding yeast *Saccaromyces cerevisiae*.

As shown in Figure 1, the CDK2T39 site is highly conserved. Earlier work had demonstrated that the homologous site in the budding yeast is indeed phosphorylated *in vivo* [143]. Cdc28, the only G_1 CDK in the budding yeast, is an essential gene that encompasses the functions of both CDK1 and CDK2 in higher eukaryotes. In addition to having simplified

cell cycle components, yeast is a good model system in which to study the cell cycle because of the relative ease with which the cells can be synchronized in G_1 and because of their short doubling time. A centromeric, non-integrating, plasmid containing the *CDC28* sequence was provided to us from Dr Marshak's lab. Using site directed mutagenesis, the Cdc28-S46 site was converted to pRS415-cdc28-S46A and pRS415-cdc28-S46E. These vectors were then verified by sequencing and used to transfect yeast cells (see strains Table 2).

Cdc28	MATa *CDC28::NAT*[WT *CDC28* in pRS415] *ade1 leu2-3, 112 his2 trp1-1 ura3Δns bar1Δ cln2::CLN2-3xHA::URA3* pRS415-*CDC-28*
Cdc28-S46A	MATa *CDC28::NAT*[*CDC28-ala* in pRS415] *ade1 leu2-3, 112 his2 trp1-1 ura3Δns bar1Δ cln2::CLN2-3xHA::URA3* pRS415-*cdc28-S46A*
Cdc28-S46E	MATa *CDC28::NAT*[*CDC28-glu* in pRS415] *ade1 leu2-3, 112 his2 trp1-1 ura3Δns bar1Δ cln2::CLN2-3xHA::URA3* pRS415-*cdc28-S46E*

Table 2. The CWY626 yeast strain was used as the parental strain for derivation of the Cdc28 genotypes used in the present study

Following selection, the chromosomal copy of the *CDC28* gene was targeted by PCR-mediated gene disruption. The yeast strains were again selected and PCR analysis was used to verify that the chromosomal copy of the *CDC28* had been indeed knocked out. Once verified, these strains were grown in glucose-containing media until early log phase and then treated with alpha factor to synchronize the cells in

early G_1. Cells were released by washing with pre-warmed media devoid of alpha and aliquots were taken at specific intervals for cell cycle analysis by Sytox green staining and flow cytometry (Figure 12). We observed a small, yet significant and highly reproducible decrease in the time from alpha factor release to S phase entry in the strain containing cdc28-S46E. The strains with Cdc28 and cdc28S46A had similar S phase entry times. This shortening of the G_1 phase was not followed by an increase in doubling time or cell growth (data not shown) which were identical in all of the strains. The lack of growth advantage for the cells containing cdc28-S46E may be the result of a compensatory pause in G2 as has been observed for other cycle active mutants that shorten G1 progression[144]. Our data partially supports this, in that the cdcS46E strain dwells in G_2 phase until the strains bearing the other Cdc28 isoforms reach G_2.

Despite its lack of effect on proliferation, the shortening of G_1 phase due to the phosphomimetic alteration in the cdc28-S46E-bearing strain is a novel discovery and points to a novel mechanism contributing to CDK regulation.

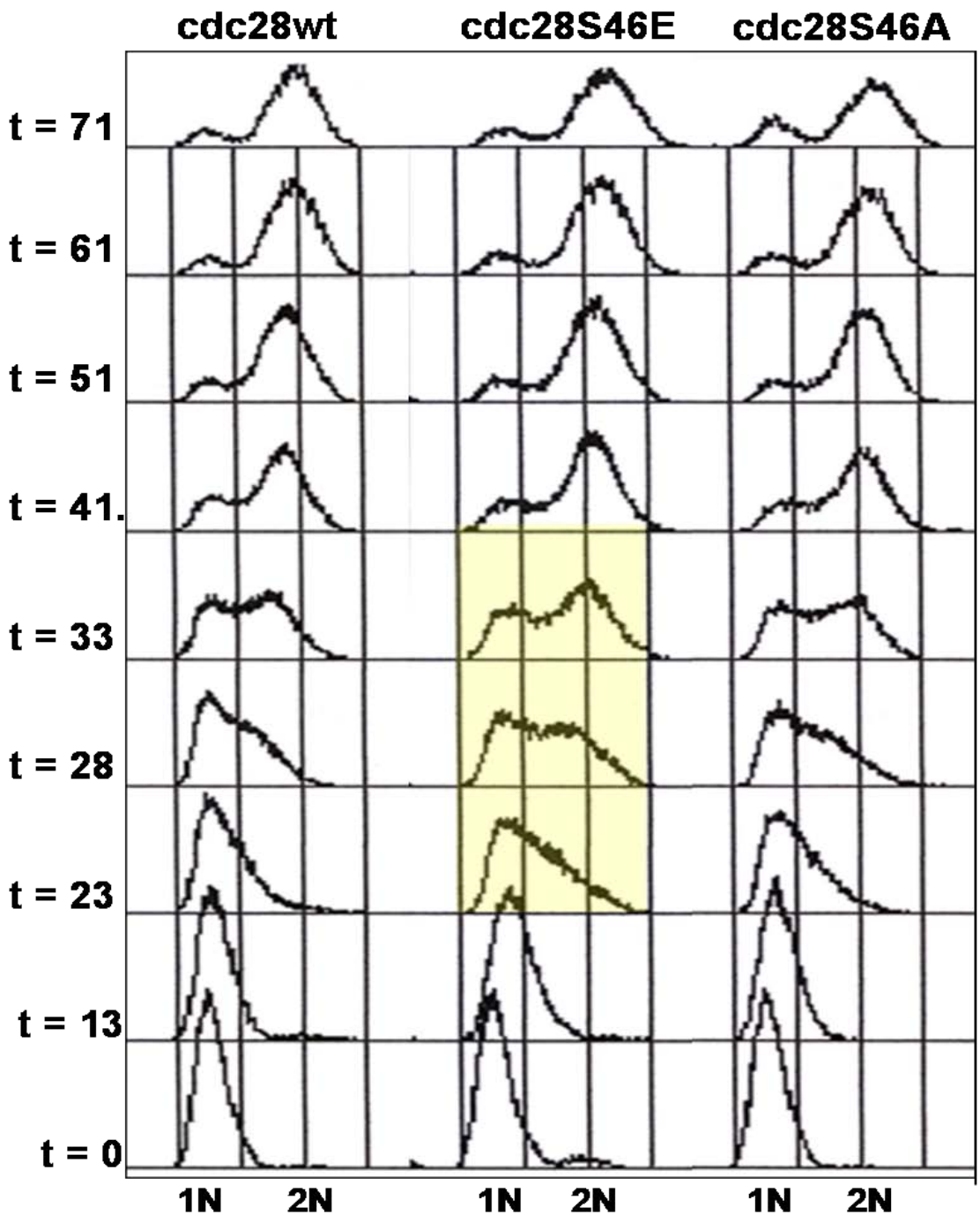

Figure 12. Cell cycle progression of *Saccharomyces cerevisiae*. Strains bearing the indicated CDC28 alleles were arrested in G_0 by addition of 50 ng/ml alpha factor for two hours and released into cell cycle. Cells were collected for cell cycle distribution analysis by flow cytometry at the indicated times. Shaded box indicates time points of greatest variance between strains.

Next, we assayed how the Akt phosphorylation site Cdc28 in *S. cerevisiae* affects Cdc28 catalytic activity. Lysates from early log phase cells from each of the 3 strains were used to immunoprecipitate Cdc28 and compare the histone H1 kinase activity of wild-type Cdc28 versus the cdc28-S46A and cdc28-S46E mutants. When equal amounts of cdc28 were immunoprecipitated from each strain (Figure 13, top band shows Cdc28 input), the kinase activity of cdc28-S46E was significantly higher than that of Cdc28 wt and cdc28-S46A. This increased catalytic activity cdc28-S46E in asynchronous lysates is consistent with our finding that the cdc28-S46E-bearing cells progress more rapidly from alpha factor release to S phase.

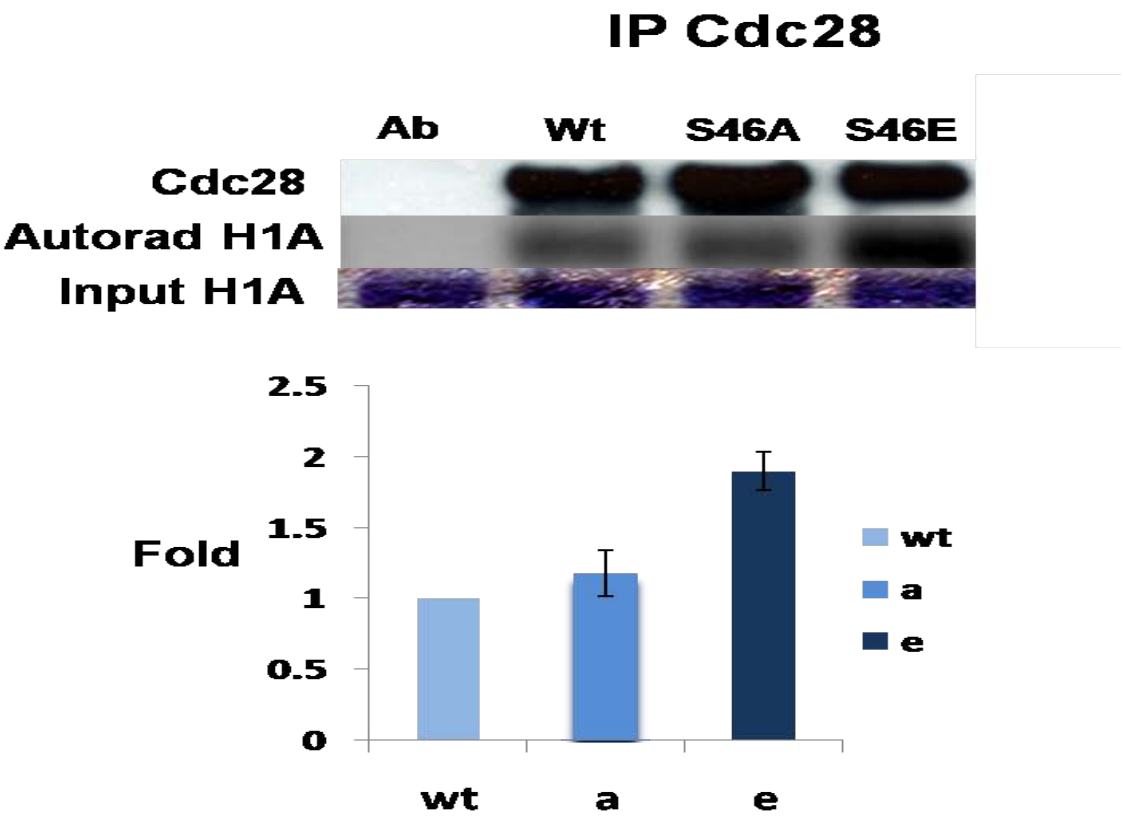

Figure 13. cdc28-S46E has higher kinase activity than Cdc28. Histone H1 kinase activity of stains bearing Cdc28, cdc28-S46A and cdc28-S46E. Exponentially growing cells were lysed and Cdc28, cdc28-S46A and cdc28-S46E mutants were precipitated from equal amounts of cell lysate using an anti-Cdc28 antibody as described in Materials and methods and assayed for Histone H1 kinase activity as described. **(A)** Immunoblotting of the precipitates used to determine Cdc28 kinase activity shows that an equal amount of Cdc28 was present in all reactions (top band), radioactivity in Histone H1 (middle band), and Coumassie stain of H1 substrate used in the reaction (bottom band) **(B)** Densitometric analysis of bands obtained from autoradiograph. Bars reflect the average of three individual experiments.

We next compared the activity of cdc28-S46E across the cell cycle compared to Cdc28. At every time point assayed, cdc28-S46E showed higher kinase activity than observed in cells with wild type Cdc28 (Figure 14). It is noteworthy that both the timing and extent of accumulation of Cln2 were increased in the cdc28S46E strain, consistent with the know effect of Cdc28 activation to phosphorylated and stabilize this Cln. Although we only immunoblotted for Cln2, one cannot discount that some of the catalytic activity of Cdc28-Cln complexes toward histone H1 could come from Cdc28 bound to other CLNs – such as CLN1 and CLN3.

Altogether we have shown that CDK activity is regulated by a novel site which can be acted upon by Akt. This, in turn, is a novel mechanism through which extra cellular mitogenic signals can influence cell cycle progression. Phosphorylation of this site is an early event in cell cycle progression. This phosphorylation alters the rate of cyclin binding and shortens the G_1 transit time.

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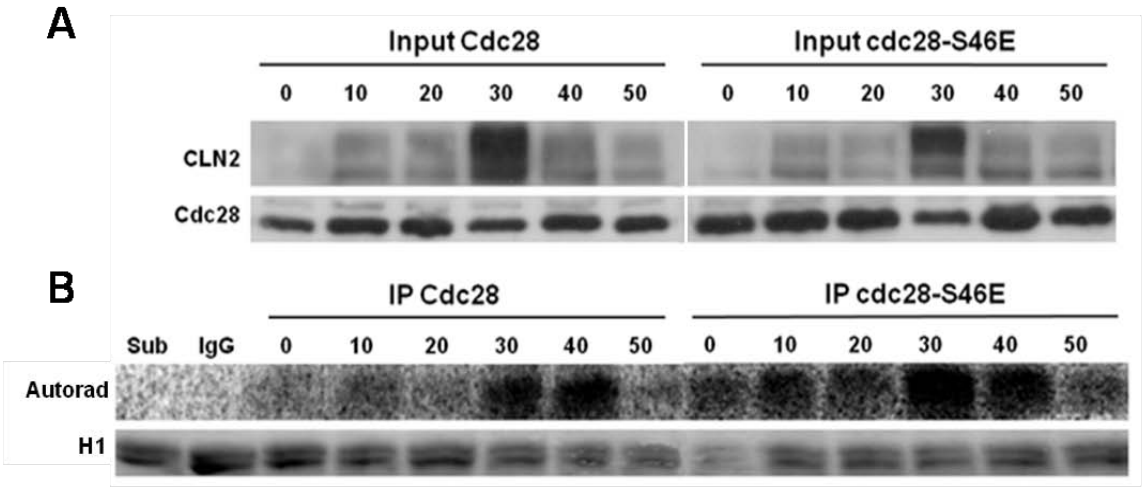

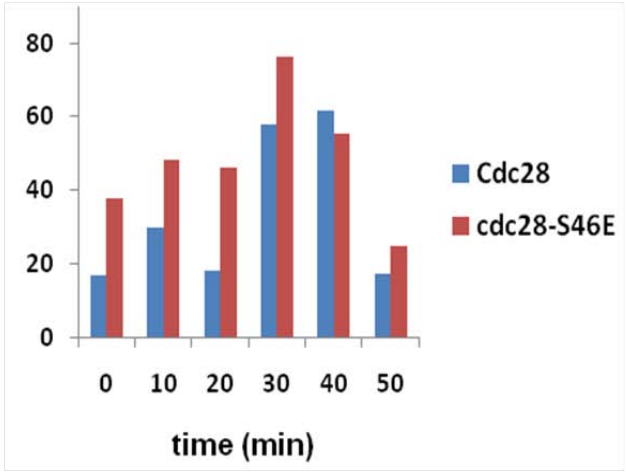

Figure 14. Cln2 levels and Cdc28 kinase activation across cell cycle. Yeast strains containing Cdc28 and cdc28-S46E were grown to early log phase and then treated alpha factor for 4 hours. G_0 cells were released into cell cycle by washing with pre-warmed media. Culture samples were taken at the indicated times and flash frozen in liquid nitrogen. **(A)** Immunoblots show the periodicity of CLN2 expression across cell cycle (top) and the relative amounts of Cdc28 (bottom). **(B)** Precipitation of equal amounts of protein from the two strains for Cdc28 IP/kinase assays show that cdc28-S46E activation precedes that of Cdc28WT and has reaches higher peak kinase activity during G1 progression. **(C)** Quantitation of radioactivity in H1 kinase substrate from kinase assays of Cdc28WT versus cdc28S46E.

Chapter 4

DISCUSSION

The present study has revealed a novel mechanism through which the PI3K pathway may act to promote G1 cell cycle progression through phosphorylation of T39 in CDK2. This PI3K-dependent phosphorylation of CDK2 at an AKT substrate motif surrounding T39 may occur in both normal and malignant cells. PI3K activity is ubiquitous to both cell types and has been shown to be required for G1 to S progression in cell of lymphoid, fibroblast and epithelial origins[141]. Deregulation of this particular mechanism may be particularly germane to cancer, since the frequent activation of the PI3K pathway seen in many cancers [133] would drive CDK2 T39 phosphoryation to accelerate G1 progression.

According to the classical model of cell cycle progression, the timed sequential activation of different cyclin-CDK complexes drives a cell to replicate its DNA and ultimately divide into two. The phosphorylation of specific substrates is critical in maintaining the ordered sequence of events required for DNA replication and subsequent chromosomal segregation. Recent genetic experiments however have challenged the notion that specific cyclin-CDKs can only drive defined phases of the cell cycle.

Although activation of CDK4 and CDK6 normally precedes that of CDK2 in G1, knockout studies in mice have shown that

these kinases are not essential for G1 cell cycle progression in most cell types. Mouse embryos develop normally until mid gestation without all interphase CDKs [89]. Pertinent to the present work, CDK2, whose disruption causes G1 arrest in somatic cells[145] [146] was found to be dispensible for G1 progression in murine embryogenesis [84, 85]. It is noteworthy that of all the CDKs, CDK1 appears essential. Knockout of CDK1 is not compensated by the presence of interphase CDKs. *CDK1*$^{-/-}$ cells are not viable and embryos do not develop beyond the two-cell stage [89]. Moreover, replacement of *CDK1* by *CDK2* using homologous recombination also results in early embryonic lethality, indicating that CDK1 cannot be compensated for by CDK2, even when expressed from the CDK1 locus [90].

A similar pattern was observed in cyclin knockout experiments. Whereas ablation of individual, or all members of different G1 cyclin families lead to tissue specific developmental defects [147] [86] [87], knockout of S-G2-M cyclins, cyclins A2 and B1 lead to early embryonic lethality [148] consistent with their requirement for proper CDK1 activation [149]. Altogether, these observations imply that CDK1 is sufficient to drive cell division in most cellular lineages. However it would be wrong to suggest

that interphase CDKs are dispensable for the faithful division of all cell types.

Regardless of the function that interphase CDKs may have during embryogenesis in genetically modified mice, data from somatic cells, many of which were lines derived from cancerous tissues, suggest something different. While in cultured cells derived from glioblastomas and osteosarcomas, inhibition of CDK2 prevents proliferation [146] [150], $CDK2^{-/-}$ mice do not show alterations in their brains or connective tissues [84]. Whereas the developing embryo appear capable of using CDK1 to compensate for the genetic lack of CDK2, disruption of CDK2 action in somatic cells has major consequences and results in cell cycle arrest or death. Data from cultured cells in which CDK2 and its partner cyclins, cyclins E and A were inhibited, indicate that these cells are dependent on normal function of G1 CDKs for cell cycle progression. Antibody microinjection experiments indicate that interference with either cyclin E1 action in normal fibroblasts [151], or cyclin A in Hela cells [152] leads to a loss of cell cycle progression. Similarly, depletion of CDK2 in cultured fibroblasts [153] and the expression of a dominant negative form of CDK2 both led to G1 arrest [146].

Malignant tumor progression appears to select for robust CDK activities during cancer development. Deregulation of CDK4 and CDK6 activities have been implicated in a wide variety of tumors (including sarcoma, breast, lymphoma and melanoma) [154] [84]. *CDK2* gene amplification and CDK2 overexpression have been documented in primary colorectal [108], lung [110] and ovarian carcinomas [111]. Misregulation of D-type cyclins and overexpression of E-type cyclins are common features in many types of tumors [91]. More recent data suggests that aberrant activation of specific interphase CDKs may indeed be required for neoplasia. CDK4-null mice, unlike their wild type counterparts, do not develop skin tumors induced by Myc [155] and are resistant to mammary tumors expressing Erbb2 and Hras under the control of the mouse mammary tumor virus promoter [156] [157]. The data on cell cycle deregulation and cancer suggests that G1 CDKs could indeed be a target for therapeutic treatment. This possibility, however, requires a better understanding of the regulatory mechanisms that underlie CDK function.

The Akt substrate motif is conserved in other CDKs (including CDK1 and CDK4), indicating that aberrations in PI3K signaling could potentially augment not only CDK2 activation but also promote the activation of other CDKs and thereby have global effects on both G1 and G2-M

progression. The specific roles of phosphorylation of homologous sites on activation of other CDK complexes has not been assayed in our study but may follow mechanisms similar to those described herein for CDK2.

The present study does not establish that CDK2T39 is an exclusive target of AKT. We observed that Akt and CDK2 form a complex in cells and that CDK2 can be phosphorylated at T39 by active AKT *in vitro*. The phosphorylation of CDK1 in the homologous site (CDK1-S39) had been previously described in human cells although the authors did not define the timing of this event nor how it may affect the cell cycle progression[158]. The S39 site in CDK1 was shown to be phosphorylated in vitro by casein kinase II (CKII) [158]. Although CKII activity is periodically activated in early G1 and this kinase could play a role in T39 phosphorylation in mammalian cells [159], CKII activation following serum stimulation is not in phase with the increase we observed in CDK2pT39. CKII activity peaks within 30 minutes of serum activation and returns to basal levels within two hours [159].

Our data shows that CDK2 forms a complex with Akt and is phosphorylated by AKT in vitro. CDK2 reacts with an antibody that detects phosphorylated AKT products. This phosphorylation is absent in quiescent MCF-7 and increases

rapidly upon mitogenic stimulation and is rapidly lost upon PI3K pathway inhibition. Of particular interest was the temporal correlation between this phosphorylation event, cyclin-CDK2 binding the accumulation of CDKpT160 and CDK2 activation.

The activation of AKT, as observed by phosphorylation at AKTS473, preceded and was temporally linked with the phosphorylation at CDK2T39. Upon mitogen stimulation of quiescent cells, T39 phosphorylation appears to precede both T160 phosphorylation of CDK2 and is association with cyclin E. Drug induced PI3K inactivation caused a rapid loss of CDK2T39 phosphorylation, that preceded the loss of CDK2pT160 and disassembly of Cyclin E-CDK2 complexes, despite no loss of cyclin E levels in the LY294002 treated cells.

Our in vitro assembly data and the immunoprecipitation data obtained from $CDK2^{-/-}$ MEFs transfected with CDK2wt, $CDK2^{T39E}$ and $CDK2^{T39A}$ also suggest that this phosphorylation event affects either the formation or stability of cyclin-CDK complexes. The discordance between the *in vitro* data (which indicates that the rate of cyclin binding is faster, although the total binding reached is similar) and the cellular data (which indicates differences in steady state abundance of cyclin-CDK complexes) may reflect the

additional effect of CDK activation upon cyclin stability in cells in vivo. It is possible that alterations in proteolytic degradation or expression of cyclin (an event frequently seen in transformed cells) triggers an accumulation cyclins and therefore we were able to observe a greater amount of cyclin bound to CDK2 in the CDK2-null MEFs transfected with T39E. Thiago need to think about this since the CLN2 in S46 E had a LOWER steady state level

In order to avoid any compensatory mechanisms that could arise in transformed mammalian cells and CDK2 null MEFs, we used the yeast model system to determine if mutations affecting cdc28S46 had a cell cycle phenotype. Previous studies using in S. cerevisiae had demonstrated Cdc28S46 phosphorylation in vivo [143]. Mutation converting Cdc28S46 to alanine reduced cell volume and protein content, but a role for this site in cell cycle progression had not been defined. We observed a slight but highly reproducible shortening of the G1 phase in the strains containing Cdc28S46E. This shortening of the G1 phase did not give these cells a growth advantage, possibly due to triggering a morphogenesis checkpoint. Asynchronous yeast lysates, as well as lysates from time points collected after release from quiescence indicated that the cdc28S46E had a greater catalytic activity than Cdc28WT, as previously published[143].

The steady state levels of Cln2 rose earlier in the cdc28S46E strain, but reached lower peak levels despite higher peak cdc28 catalytic activity. This may reflect the effect of Cdc28/Cln2 kinase to promote Cln2 degradation. The in vitro and in vivo date presented support a model in which phosphorylation of CDK2 at T39 and of Cdc28 at S46 may promote more rapid assembly with cyclins, and modulate the timing or stabilization of T160 phosphorylation. This novel regulation mechanism through which phosphorylation of G1 CDKs, specifically but perhaps not limited to CDK2, would influence the duration of G1 phase.

Our current understanding of CDK activation and cell cycle progression contains very little in terms of post translational modifications that alter the affinity of CDKs for cyclins. To date, the known phosphorylations events in CDKs alter the cyclin-CDK catalytic activity. Across the cell cycle, the phsphorylations in CDK2Y15 and CDK2T160 increase through G1 [57, 160]. This may be due to the increase in activity of wee1 kinases as a response to mitogenic stimulation and the constant activity of CAK [30, 67]. Although CDK2 can be acted upon by CAK when in its monomeric form, the accumulation of CDK2pT160 is a direct function of cyclin binding as cyclin association prevents dephosphorylation of the T-loop. Fisher et. Al. have put

forth a model whereby CDK2 is phosphorylated at CDK2T160 prior to cyclin binding [64]. The observation that cyclin-CDK binding is required for nuclear import of the complex raises a problem with the model of Fisher et al. Since CDK-activating kinases *in both budding and fission yeast* appear to localize to the nucleus, it would be difficult for the CAK complex to phosphorylate CKD2 complexes prior to its binding to cyclins, since the CDK2 monomers appear to be largely cytoplasmic [161]. We envision CDK2T39 phosphorylation serving as a trigger mechanism that facilitates cyclin-CDK assembly and possibly also nuclear import. Previous work has shown that cytoplasmic mislocalization of active cyclin A-CDK2 leads to apoptosis [162]. The PI3K pathway is a strong mitogenic and anti-apoptotic signal. Thus it is possible that constitutive activation of the PI3K pathway in cancers inhibits, or alters the rates of nuclear export versus import, of cyclin-CDK complexes, thereby preventing apoptosis and driving the cells toward S-phase. This way, extracellular insults such as gamma irradiation would not cause a cell cycle checkpoint and possibly lead to genetic instability.

Future Directions

The findings of my thesis work suggest that activation of the PI3K pathway, and its downstream effector AKT, leads to the phosphorylation of CDK2T39. This, in turn, causes CDK2 to form complexes with its cyclin partners more rapidly and results in an active kinase that has a greater catalytic activity. In yeast cells, the phosphomimetic cdc28S46E has a shorter G1-S phase transit time. There are several unresolved questions that arise from this work that warrant further investigation in the Slingerland lab. The following proposed experiments would extend my current line of investigation and further explore the mechanisms whereby the PI3K pathway regulates CDK2 activity.

Inquiry 1: How does CDK2T39 phosphorylation affect subcellular CDK2 localization?

The experiments above indicate that the phosphorylation at CDK2T39 occurs prior to the increase in phosphorylation at CDK2T160 as cells progress from G0 to S phase. In addition, PI3K pathway inhibition caused a rapid loss of phosphorylation at CDK2T39 which preceded the loss of the CDK2T160 phosphorylation. This temporal link between the two sites, coupled with the shorter G1 to S phase transit seen in yeast strains carrying a T39 phosphomimetic mutation in the CDK2 homologue, cdc28, suggest that

phosphorylation at the T39 site positively regulates the subsequent action of the CDK activating kinase (CAK) on CDK2. This may occur directly through a conformation effect on CDK2. Alternately, the greater action of CAK on CDK2 may be driven by a T39-dependent translocation of CDK2 to into the nucleus.

The size of CDK2 (34 kDA) and cyclins E and A (54 and 60 kDA , respectively) would permit them to translocate freely through the nuclear pores between nucleaus and cytoplasm. However, the cyclin-CDK2 complexes localize to the nucleus in late G1 in parallel with their periodic catalytic activity, indicating that Cyclin-CDK2 localization is actively regulated. CAK is predominantly nuclear localized thoughout the cell cycle; cyclins E and A accumulate in the nucleus in late G1 and S phases, while CDK2 is both nuclear and cytoplasmic (Refs). CDK2 substrates are mostly nuclear proteins. Chiefly among them are histone H1, proteins involved in initiation of DNA synthesis and the retinoblastoma protein (ref). Thus, if CDK2 T39 phosphorylation enhanced the rate of nuclear import of CDK2, or cyclin-CDK2 complexes toward their sites of action in the nucleus, this could trigger a shortening of G1 to S phase progression.

Experiment 1: Does the phosphomimetic mutant cdc28S46E show enhanced nuclear localization compared to wild type? We have created yeast strains that contain Cdc28wt, cdc28S46A and cdc28S46E. These strains could be synchronized in G1 by alpha factor synchrony experiments and collected at different time points as they re-enter the cell cycle. The localization of Cdc28 and the mutant cdc28 proteins could be visualized by immunofluorescence. We would expect to see that the cdc28S46E mutant may accumulate in the nucleus earlier and or at higher levels than would be observed for Cdc28 and cdc28S46A.

Experiment 2. Does CDK2pT39 increase binding to importin-α/β? The nuclear import of cyclin E-CDK2 (and of cyclin E alone) has been shown to require binding to the import proteins importin-□ and importin-β (Moore et al., 1999). CDK2 lacks a nuclear import signal. Its translocation in to the nucleus is in part mediated by its binding to cyclin E, because that latter contains a nuclear localization signal that mediates importin binding. One mechanism whereby T39 phosphorylation on CDK2 may enhance nuclear localization could be via an enhanced interaction between cyclin E-CDK2 complexes with the importin proteins. Thus, it would be of value to test if T39-phosphorylated CDK2 (CDK2pT39) may have a greater affinity or enhance the stability of

association with the importins compared to non-phosphorylated CDK2.

THIAGO: PLEASE RE-READ THE MOORE KORNBLUTH PAPER> I THINK THE IMPORTINS BIND ONLY TO THE CYCLIN E??? You also need to read some reviews on mechanisms of nuclear import and export in preparation for the defense- See our Connor paper This could be tested by comparing mixtures of recombinant cyclin E with either CDK2wt, CDK2T39A or CDK2T39E proteins already present in the lab in an in-vitro binding assay using commercially available, recombinant, GST-tagged importin-□ or β. Alternatively, we could pre-treat CDK2wt with AKT and then use it in binding assays with importin-□ or β in the presence of cyclin E. The phosphorylation of CDK2 at T39 may not only enhance cyclin E-CDK2 complex association, but may also enhance to binding of the cyclin E-CDK2 complex to importin□. Controls in these experiments would include binding reactions of the different CDK2wt, CDK2T39A or CDK2T39E proteins to the importins alone, in the absence of cyclin E and binding of the cyclin E to importin alone, in the absence of CDK2. An increase in the steady state levels of importin-□ or β bound to recombinant cyclin E-bound CDK2T39E or AKT pre-treated CDK2wt, compared to CDK2wt, untreated with AKT,

would indicate a mechanism whereby cyclin E-CDK2 complexes could indeed accumulate in the nucleus more readily.

Experiment 3: Effects of CDK2pT39 on nuclear import in vitro. We could assay the in vitro nuclear import of recombinant CDK2-cyclin or CDK2 alone into isolated nuclei as follows. Cells are briefly permeabilized by treating with digitonin which permits escape of cytosolic proteins, leaving behind "bare nuclei." These nuclei could then be reacted with recombinant CDK2, recombinant importin-α/β, RanGDP and an ATP generating system (Adams, 1992). Following incubation for specific intervals, we would verify the extent of nuclear CDK2 import by separating the nuclei from the supernatant via centrifugation, and assaying for imported CDK2 protein by lysis of the nuclei followed by western blotting. Here too, we could use either recombinant CDK2T39E or we could pre-treat recombinant CDK2 wt with AKT and compare its rate of import to non-treated CDK2, both in the presence and absence of recombinant cyclin E. We anticipate that the cyclinE-CDK2 complex will be imported more rapidly when the CDK2 is either pre-treated with ATK or the phosphomimetic variant is used. We anticipate that CDK2 import will be affected by the T39 status only when the import assays are carried out

in the presence of cyclin, since only the latter is capable of binding the importin machinery.

THIAGO: Think about how you would distinguish rate of import differences versus an effect of T39 on the stability of the cyclin E-cdk2 complex, since the cyclin E NLS is what binds to the import machinery.

Significance: These assays may illuminate further the mechanisms through which the PI3K pathway promotes CDK2 activation in higher eukaryotes.

Inquiry 2: Does CDK2T39 phosphorylation may it a better substrate for CAK ?

Full activation of CDKs requires T160 phosphorylation at the T-loop via CAK. Our data indicates that phosphorylation of CDK2 at T39 precedes that on T160 in cells and induces a faster rate of cyclin-CDK complex formation in vitro. Moreover, the phosphomimetic mutation of the yeast CDK homologue at this site, cdc28S46E, has a higher catalytic activity than the Cdc28wt. In the case of CDK1, CAK action requires prior cyclin binding. In contrast, CDK2 is thought to be phosphorylated by CAK as a monomer, but cyclin binding protects the T160 site from dephosphorylation. Since we have observed that the appearance of the CDK2pT39 precedes that of CDK2pT160 during G0-S phase progression, this raises the possibility

that T39 phosphorylation may condition the CDK2 for action by CAK. T39 phosphorylation may not only promote more stable cyclin association, but may also modify the conformation of the CDK2 monomer to permit more ready phosphorylation by CAK.

Experiment 1: Is the steady state binding of CAK and CDK2 altered by mutations at T39? Some kinases are known to form transient complexes with their substrates (Brazil, 2002). Thus, we would like to determine if the CAK-CDK interaction would be altered by CDK2T39 mutations. To do this, we could transiently transfect MCF-7 cells with HA tagged CDK2 constructs and immunoprecipitate using an anti HA antibody. These precipitates could then be used to probe for one of the subunits of CAK (CDK7, Mat A, Cyclin H). Changes in the steady state binding could be indicative of a greater affinity between CAK and CDK2 or a greater stability of the complex, once formed.

Experiment 2: Does CDK2T39E serve as a better in vitro CAK substrate? This could be tested by performing a kinase assay using recombinant, active CAK expressed from bacculovirus in insect cells and recombinant CDK2 as a substrate. Differences in the rate or amount of CDK2pT160 product formed from CDK2T39E, CDK2T39A and CDK2wt substrates would indicate either differences in CAK

affinity for the substrate or efficiency of the reaction. Additionally, we could pre-treat CDK2wt with AKT and test if the rate or extent of the action of CAK on CDK2 was affected.

Cak1p is the *S cerevisieae* homologue of the human CAK that phosphorylates Cdc28 at T169 (the site homologous to T160 in human CDK2). As an alternative strategy, one could immunoprecipitate Cdc28, cdc28S46E and cdc28S46A from alpha factor treated cells and compared these substrates in a Cak1p kinase assay. Long term treatment with alpha factor would abolish most of the T169 phosphorylated cellular Cdc28 such that the immunoprecipitated complexes could serve as substrate in a Cak1p kinase assay.

Significance: Determining if CDK2T39 phosphorylation plays a role in CAK function would be instrumental in elucidating the mechanisms linking both T39 and T1260 phosphorylation events and how activation of the T39 phosphorylation event shortens the G1 to S phase transit time

Inquiry 3: How does expression of the more catalytically active *cdc28S46E* allele affect CLN2 stability and phosphorylation?

Our data using the budding yeast model system showed that cdc28S46E had a higher catalytic activity than Cdc28.

Additionally, we repeatedly saw that the cdc28S46E strain had a lower steady state level of CLN2, even though the levels of Cdc28 and PGK1 (a cytoplasmic protein used as loading control, data not shown) were similar in both lysates. Since the CLN2 degradation is activated by its phosphorylation by CLN2-cdc28 (lanker; 1996), this might explain the lower levels of CLN2 in the yeast expressing cdc28S46E. Thus, we would like to determine if the increased catalytic activity of the phosphomimetic mutant could be turning on a negative feedback loop and thereby decreasing the levels of CLN2 present in the cells.

Experiment 1: Does the half life of CLN differ between Cdc28 strains? To test this, we would perform a cycloheximide chase and determine the half life of Cln2 in Cdc28, cdc28S46A and cdc28S46E strains. We could assay the loss of Cln2 protein by recovering lysates at intervals after cycloheximide treatment and immunoblotting the lysates for HA (a tag that was added to the *CLN2* gene). Alternatively, we could pulse label the cells by treating them with [^{35}S]-methionine, transfer to chase media containing cold methionine and then assay the decay of incorporated radioactivity in HA-Cln2 at intervals thereafter by HA-immunoprecipitation, resolution on SDS-PAGE and autoradiography of dried gels.

Experiment 2. Would proteasome inhibition restore the concentration of CLN2 proteins to similar levels in Cdc28 and cdc28S46E? We postulate that the increases activity of cdc28S4E is triggering a negative feedback loop leading to degradation of its Cln2 partner. If this is indeed the case the treatment with MG132 should diminish the difference if Cln2 concentration between the two strains. We could treat either asynchronously growing cells, or cells that are 20 to 30 minutes into the cell cycle from an alpha factor release with a proteosomal inhibitor (MG132). The proteosomal inhibitor would inhibit the degradation of Cln2 and therefore we could observe if indeed the different levels of Cln2 are due to increase degradation.

Significance: It is well established that for several G1 cyclins, including both cyclin E and Cln2, degradation is triggered by CDK-mediated cyclin phosphorylation (Wittenberg, science, 1996 refs). The finding that cdc28S46E has a lower Cln2 concentration than that in the Cdc28wt strain, provides an in vivo validation of our data showing that cdc28S46E has greater catalytic activity.

In several cancers, it has been shown that G1 cyclins, cyclin D1 and cyclin E are stabilized through mechanisms that are not entirely clear. Moreover, CDK2 is often overactivated in cancers by constitutive receptor tyrosine

kinase activation (such as Met, Her2 or EGFR), activating mutations of the catalytic component of PI3K, *PIK3CA*, or of PI3K effectors including *AKT*. Cancers may select for cyclin overexpression or for greater cyclin stability to allow the cancer cell to overcome the negative feedback look that occurs after a threshold of CDK activity is achieved. This would permit the neoplastic cells to maintain an abnormally high mitogenic signal and enforce cells accelerated G1 cell cycle transit.

Inquiry 4: Do changes in T39 phosphorylation have different consequences in cancer-derived versus normal somatic cells of finite lifespan?

One of the biggest confounding factors in the study of CDK2 in mammalian cells is the use of either immortalized or cancer-derived transformed cells to study a phenotype that, in yeast, is relatively modest. Deregulation of cell cycle controls and increased G1-S phase transit is a universal hallmark of cancer cells. Transformed cancer-derived cell cultures have undergone a selection yielding a very robust cell cycle. CDK2 activation is frequently observed in cancer cells (Chu Nat Revie Cancer 2007), raising the possibility that these lines are “oncogene-addicted” to activated CDK2. This possibility is supported by the observation that in cultured cells derived from

glioblastomas and osteosarcomas, inhibition of CDK2 prevents proliferation [146] [150], while cell cycle proliferation in embryogenesis is not perturbed in CDK2-/- mice and these animals do not show alterations in their brains or connective tissues [84].

Given the frequent deregulation of the PI3K pathway in human cancers, one might expect that the phenotype of a CDK2T39E may be lost in a cancer cell line. The following are proposed to investigate the consequences of T39 phosphorylation in malignant versus normal finite lifespan cell types.

Experiment 1: Is there a difference in the timing of CDK2 T39 phosphorylation and steady state CDK2-AKT binding between transformed and primary cells? In a first set of experiments, I propose to compare the timing of CDK2T39 phosphorylation during G1 to S phase in a series of cancer-derived cell lines (breast and lung) and normal finite lifespan epithelial cells from breast and lung tissues. I will chose cancer lines that can be synchronized in quiescence by growth factor or serum deprivation. These lines will be compared with regard to the kinetics of the T39 phosphorylation of CDK2, the duration of G0-S phase cell cycle progression, and the timing of cyclin-CDK2

binding and CDK2 activation. I would anticipate that many of the malignant lines would show a shorter G1-to S duration and more rapid onset of T39 phosphorylation on CDK2, cyclin-CDK2 complex formation and activation, and that this would be directly proportional to their degree of oncogenic AKT activity compared to normal epithelial cells so the same tissue origin.

I would also test if extent and timing of the steady state binding between AKT and CDK2 differs between primary cells and transformed cells. As AKT is more active in many malignantly transformed lines, I expect the steady state binding between these two molecules would be lower in primary cells.

Experiment 2. **Does replacement of *CDK2* with *CDK2T39E* have different consequences in a malignant line compared to finite lifepan epithelial cells?** Finally, it would be of interest to compare the consequences on G1-S transit time when cellular CDK2 is replaced by homologous recombination with CDK2T39E in somatic cells of malignant origin versus finite lifespan epithelial cells. For this, one would replace the endogenous *CDK2* gene sequence with a *CDK2T39E* encoding gene. The sister lines so derived would be compared for the effects on G1-to-S phase timing, CDK2-AKT complex formation, CDK2-cyclin-binding and CDK2 activation.

One would expect that in the transformed lines, that are already driven by a constitutively activated PI34K pathway, the introduction of CDK2T39E would have little effect. In contrast, replacement of cellular CDK2 with the CDK2T39E would shorten the G1 transit time in normal finite lifespan epithelial cells, by increasing cyclin-CDK2 complex formation and CDK2T160 phosphorylation.

Significance: In the context of cancer cell lines, in which PI3K is already oncogenically activated or in which CDK2 is already activated by loss of the CDK inhibitors p21 or p27, or by CDK2 gene amplification, the effects of a phosphomimetic CDK2T39E mutation, or indeed loss of potential to phosphorylated T39 in a non-phosphorylated CDK2T39A may not be readily apparent. A phosphomimetic mutation at CDK2T39 that has a modest effect to facilitate cyclin-CDK2 complexing and/or T160 phosphorylation in normal cells, may not be readily apparent in cancer cells that are progressing through cell cycle at already maximal speed.

Experiment 3 What would happen to CDK2 upon transient transfection of primary cells with constitutively activated myr-AKT or AKT^{DD}? By transiently transfecting a vector encoding a constitutive active AKT into primary cells I could determine if there are any differences in CDK2T39

phosphorylation, CDK2 subcellular localization and activation. I expect that transiently transfected cells would have an increase in CDK2pT39, increased nuclear CDK2, greater cyclin-CDK2 steady state levels and higher catalytic activity.

Significance: Demonstrating that the timing of CDK2T39 phosphorylation and CDK2 subcellular localization is affected by constitutive activation of Akt would provide further support for the notion that oncogenic deregulations in the PI3K pathway can push transformed cells prematurely through G1 S of the cell cycle in response to, and indeed independently of mitogenic signals. This, in turn may lead to chromosomal aberrations which could promote neoplastic tumor progression.

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